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Award Number: DAMD17-98-1-8466

TITLE: Prostate Tumor Antigen Discovery: Development of a Novel Genetic Approach

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REPORT DATE: December 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE December 2001	3. REPORT TYPE AND DATES COVERED Final (1 Jul 98 – 30 Nov 01)

4. TITLE AND SUBTITLE
Prostate Tumor Antigen Discovery: Development of a Novel Genetic Approach

5. FUNDING NUMBERS
DAMD17-98-1-8466

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

13. ABSTRACT *(Maximum 200 Words)*

The overall objective of this study was to determine the *in vitro* immunogenicity of products of prostate tumors that could potentially be used as tumor vaccines. To achieve this goal, the following specific tasks were undertaken: (1) detection of recall T cell responses to known tumor antigens, (2) optimization of antigen delivery to and presentation by dendritic cells (DC), and (3) develop oligonucleotide-derived tumor antigens in cDNA libraries. T cell recall responses to prostate specific antigens (PSA) were found in patients with autoimmune prostatitis and prostate cancer showing that this protein is potentially immunogenic. CD4+ and CD8+ T cell lines established from these patients were used to identify previously undescribed HLA Class I and Class II allele-specific epitopes in PSA. Methods were developed to isolate DC precursors from peripheral blood and to use these cells to generate *de novo* immune T cell responses *in vitro*. DCs armed with PSA-generated CD4+ T cell responses, while both CD4+ and CD8+ responses were observed with DCs armed with PSA/anti-PSA immune complexes. Only CD4+ T cell responses developed with DCs transfected with RNA from LNCaP cell lines. These studies established the feasibility of using PSA as a vaccine for prostate cancer immunotherapy.

14. SUBJECT TERMS
prostate cancer, tumor antigens, immune response, dendritic cells, autoimmunity, antigen processing and transport genes

15. NUMBER OF PAGES
177

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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INTRODUCTION

The objectives of this proposal are based on the hypothesis that products of prostate cancer cells are capable of generating an immune response, and that these products can be used as an effective vaccine for adjunct therapy for this malignancy.

The immune system has the capacity to recognize products of malignant cells that have amino acid changes that are different from normal proteins or, in some instances, products that are overproduced by the malignant cell relative to their normal counterpart.

Once an immune response is established to specific cellular products, the antigen must be exhibited in the form of peptides by HLA Class I molecules on the surface of the malignant cell in order for that cell to be targeted for immune elimination. A number of studies have demonstrated that MHC Class I and Class II molecules are inadequately expressed on tumor cells and, therefore, might escape immune detection. The lack of expression of genes that encode products that generate peptides and those that transport peptides to compartments of cells where they are loaded into Class I molecules have been found in some instances to account for the lack of antigen presentation by tumor cells. We undertook studies to determine if these genes (LMP and TAP) were expressed in cultured cell lines established from primary and metastatic prostate cancer as well as in long-term prostatic cancer tissue culture cell lines.

Dendritic cells (DC) are the only antigen presenting cells that can induce a primary immune response. These cells can be isolated from peripheral blood and used to establish the capacity of the immune system to generate immune responses *in vitro*. With the development of this technology, it is possible to test potential tumor antigens for their immunogenicity *in vitro* prior to their use as immunogens in vaccine constructs. A number of different methods have been used to deliver vaccines both *in vitro* and to test their immunogenicity *in vivo*. The studies conducted and reported herein explored several mechanisms whereby antigens could be acquired by DC and used to generate T cell responses. To establish the immunogenicity of PSA in individuals with conditions where this protein is overproduced by the prostate, recall response to this protein were assessed in T cells from patients with the prostate cancer or autoimmune prostatitis.

BODY

The technical objectives of this proposal are listed as follows. Task 1. Stimulation and detection of recall lymphocyte responses to known prostate antigens. The purpose of this task was to develop and test methods for generating responsive T cell populations for subsequent antigen discovery and epitope mapping. Task 2. Comparison and optimization of antigen delivery to and presentation by myelomonocytic-derived and non-myelomonocytic-derived dendritic cells. The purpose of this task was to develop methods for priming DC to present antigens from prostate cancer cells. Task 3. Development of oligonucleotide-derived tumor antigens by screening cDNA libraries. The purpose of this task was to develop technology which would enable polynucleotides expressed by prostate cancer to serve as a means to generate immune responses and thus serve as a vaccine.

Autoimmune diseases share many of the features that might be desired in cancer immunotherapy. In order to generate an effective immune response against products of tumor cells, tolerance to these proteins must be broken and autoreactivity generated. In our studies, we demonstrated that some patients with chronic prostatitis have circulating T cells that respond to prostate antigens while no such reactivity was found in normal men (see appended manuscript by Ponniah et al. for detailed experimental procedures and results). This response was further characterized by generating T cell lines by repeated PSA stimulation using peripheral blood lymphocytes from patients with chronic autoimmune prostatitis and with prostate cancer. Responses were monitored by testing for IFNγ production in culture fluids by ELISA as well as determining T cell activation by assessing intracellular cytokine production using flow cytometry techniques as well as in ELISpot assays. The results of the studies with cells obtained from a patient with

granulomatous prostatitis is detailed in an appended manuscript which is being prepared for submission (Klyushnenkova et al.). The results are summarized as follows. The detailed HLA types were determined using molecular methods. The cancer patient and the prostatitis patient shared HLA Class I alleles B*0702 and a Class II allele DRB1*1501. Cell lines derived from these patients were used to identify allele-specific PSA peptides recognized by T cells from these individuals. This result extends a number of Class I alleles that can present PSA peptides to generate CD8+ T cell responses and establish for the first time an HLA Class II restricted epitope in this protein. In addition, B cell lines were established from the cancer patient and transfected with a recombinant clone expressing PSA. T cells from this individual responded to this EBV transfected cell line indicating that the CD8+ T cells can recognize endogenously produced PSA.

DC are the sentinel antigen presenting cell of the immune system. DC precursors can be isolated from peripheral blood. The most abundant of these precursors are products of cells in the myelomonocytic lineage (10-15% of peripheral blood mononuclear cells). These DC express combinations of CD11c, CD14, and HLA-DR. Other DC precursors are the lymphoid lineage and constitute about 1-3% of the total lymphoid population of peripheral blood mononuclear cells. The myelomonocytic precursors were selected for our studies because of their abundance in peripheral blood and their ability to preferentially generate TH1-type immune responses. Mononuclear cells were isolated from whole blood obtained by apheresis from normal donors and from patients with prostate cancer or non-infectious prostatitis. Each donor was typed for HLA Class I and Class II alleles at the DNA level. We investigated and compared several isolation techniques. This included sorting by flow cytometry, isolation of loosely-adherent cells, and by negative selection using immunomagnetic beads. All three preparations were capable of inducing *in vitro* primary immune responses to the test antigens used. However in consideration of cost of the technology, the majority of the studies were conducted by isolation of the DC by selecting loosely adherent cells. The details of these isolation procedures and results of the studies can be found in the appended manuscript entitled "Generation of CD4+ and CD8+ T Lymphocyte Responses by Dendritic Cells Armed with PSA/anti-PSA (antigen/antibody) Complex" by Berlyn et al. The technology and the results are summarized as follows. DC precursors were isolated from peripheral blood, cultured for 4 days in GM-CSF and IL4, exposed to various antigen preparations, matured with cytokines and tested for the induction of CD4+ and CD8+ T cell responses using autologous T cells. We found as have others that the timing of antigen presentation was critical for antigen uptake and subsequent generation of CD4+ and CD8+ T cell responses. DC are known to express a number of cell surface receptors that mediate antigen acquisition. We targeted several of these receptors to investigate the possibility that specific-T cell responses were favored when antigen was acquired by several of these receptors. We compared the capacity of DC to generate CD4+ and CD8+ T cell responses after exposure to prostate-specific antigen alone, prostate-specific antigen targeted to the mannose receptor or PSA targeted to the Fc receptors by combining PSA with an anti-PSA antibody. Both CD4+ and CD8+ T cell responses were observed after stimulating T cells with dendritic cells exposed to the PSA/anti-PSA complexes whereas CD4+ responses predominated over CD8+ T cell responses after stimulation with DC armed with PSA alone or PSA that had been conjugated to mannose. These results indicated that PSA presented to the DC in the form of antigen antibody complexes led to processing through pathways that generated HLA Class I mediated T cell responses, while PSA alone or PSA that had been targeted to the mannose receptor generated only Class II-mediated CD4+ T cell responses.

Products of cancer cells in the form of tumor cell lysates have been and are being used in several clinical trials of immunotherapy of several different cancers. We investigated the capacity of DC to generate CD4+ and CD8+ T cell responses to lytic products of several long-term prostate cancer cell lines, LNCaP and DU145. Two methods of disruption of these cells were used. The first was to induce apoptosis using UVB irradiation and the second a simple freeze-thaw procedure that preferentially isolates membrane components of these cells. These products were exposed to DC at various stages of maturation and T cell responses were shown to occur when the cellular products were exposed to immature DC and then matured prior to exposure to T cells. Responses generated from the UVB irradiated T cell lines occurred, however, were nonspecific and inconsistent. T cell responses to DC exposed to cell membranes were more specific. The LNCaP cell line produces PSA, while the DU145 does not. We took advantage of this differential expression of PSA to demonstrate that T cells generated by membrane preparations from the LNCaP cell line were responsive to dendritic cells armed with the PSA/anti-PSA complex.

One of the objectives of this proposal was to develop the methodology for screening oligonucleotides derived from prostate cancer/prostate cancer cell line as a means of induction of T cell responses using the DC technology. To explore this possibility and investigate this technology, we first chose to investigate transduction of DC by retroviral or vaccinia virus constructs expressing PSA. These constructs were kindly provided by Dr. Jeffery Schloom (Laboratory of Tumor Immunology and Biology, NIH, Bethesda, MD). DC were exposed to these constructs at various stages of differentiation and maturation, however, we failed to detect PSA production by these cells when culture fluids and/or transfected disrupted cells were screened in an ELISA assay. Moreover, DC exposed to these constructs failed to generate CD4+ or CD8+ T cell responses. In other attempts, RNA was prepared from the LNCaP cell line, converted to cDNA, expanded and then reconverted to RNA. Immature dendritic cells were exposed to these RNA preparations and tested for their capacity to generate CD4+ and CD8+ T cell responses. Low levels of CD4+ T cell responses were observed in these preparations and on some occasions minimal to low levels of CD8+ T cell responses. However, there was considerable inconsistency using multiple RNA preparations from the LNCaP cell lines.

KEY RESEARCH ACCOMPLISHMENTS

- CD4+ and CD8+ T cell lines and clones were generated from peripheral blood from patients with prostatic cancer and granulomatous prostatitis.

- Previously undescribed HLA-B*0702 restricted PSA peptides were identified as well as an HLA Class II allele restricted PSA response.

- Dendritic cells were isolated from precursors in peripheral blood matured with cytokines and were found to present PSA generating CD4+ T cell responses.

- CD4+ T cell responses predominant when PSA was presented by dendritic cells, while both CD4+ and CD8+ T cell responses were observed when dendritic cells were armed with PSA combined with an anti-PSA antibody.

- Targeting PSA to mannose receptors by a PSA/mannosylated product generated CD4+, but not CD8+ T cell responses.

- Dendritic cells armed with lysates of PSA producing cell line generated.

- Cell lines established from primary and metastatic cancer.

- CD4+ and CD8+ T cell responses that also recognized HLA restricted PSA peptides were shown to express antigen processing and peptide transport genes that could be upregulated by IFNγ.

- RNA prepared from the LNCaP cell line converted to cDNA amplified and retranscribed into RNA was acquired by dendritic cells and generated CD4+ T cell responses.

REPORTABLE OUTCOMES

"PSA as a candidate cell antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome." S. Ponniah, I. Arah, R. B. Alexander. The Prostate 44: 49-54, 2000

"Generation of CD4+ and CD8+ T lymphocyte responses by dendritic cells armed with PSA/anti-PSA (antigen:antibody) complexes." K.A. Berlyn, B. Schultes, B. Leveugle, A. Noujaim, R.B. Alexander, D.L. Mann. Clinical Immunology 101(3): 276-83, 2001.

Manuscript in revision. "Antigen processing and presentation gene expression in normal primary and metastatic prostate tumor cell lines." K.A. Berlyn, R.B. Bright, R.B. Alexander, D.L. Mann. Submitted to The Prostate.

Manuscript in preparation. "CD4 and CD8 T lymphocyte recognition by prostate specific antigen in a patient with granulomatous prostatitis." E. N. Klyushnenkova, S. Ponniah, A. Rodriguez, J. Kodak, D. L. Mann, R. B. Alexander.

Abstract. "Generation of prostate specific antigen (PSA)-reactive T cell lines – implications for prostate cancer immunotherapy." E. N. Klyushnenkova, S. Ponniah, D. L. Mann, R. B. Alexander.

Abstract. "Generation of human CD4 and CD8 T lymphocyte lines that recognize prostate specific antigen (PSA) from peripheral blood from a patient with granulomatous prostatitis." E. N. Klyushnenkova, S. Ponniah, A. Rodriguez, J. Kodak, D. L. Mann, R. B. Alexander.

Review. "Therapeutic antibodies for prostate cancer." B. Leveugle, D. Mann, R. Madiyalkan, A. Noujaim.

Patent Pending. "Therapeutic method and composition utilizing antigen-antibody complexation and presentation by dendritic cells." (Attorney Docket No. 107823.186).

CONCLUSIONS

Immunotherapy of prosate cancer may serve as an effective adjunct to current treatment modalities. The overall objective of this project was to identify candidate antigens that will serve as targets for prostate cancer immunotherapy and to test the immunogenicity to these antigens using an *in vitro* assay system employing the primary antigen presenting cell of the immune system, the DC. The objectives were based on the hypothesis that products of prostate cancer cells are capable of generating an immune response that will be effective as an adjunct therapy for this malignancy. There are several road blocks to developing a successful cancer vaccine. Products of tumor cells that are candidate antigens are likely to be perceived by the immune system as self and thus the individual will be tolerant to the potential antigens. Vehicles that will deliver the tumor antigen and generate immunity will be required to overcome this tolerance to self. In addition, there is extensive heterogeneity in HLA Class I and Class II alleles that are present in the population at large and thus potentially restrict the antigenic determinants that can be generated from any specific product or protein that would be used for immunotherapy. We addressed some of these issues in the specific aims of the project. These were to develop an *in vitro* technique to isolate primary antigen presenting cells and to arm these cells with antigens for the generation of CD4+ and CD8+ T cell responses. We also sought to develop reproducible methods for assessing CD4+ and CD8+ T cell responses *in vitro* since this is likely that responses by both of these cell types will be necessary in order for effective anti-tumor immunity. To achieve these goals, methods for isolating DC from peripheral blood sources were developed and their capacity to generate *de novo* immune responses were tested. We developed several assays for T cell responses including the ELISpot and the production of intracellular cytokines by CD4+ and CD8+ T cells on cognate antigen recognition. We demonstrated the capacity of DC to acquire PSA presented in the form of whole proteins and in combination with the monoclonal anti-PSA antibody. DC armed with PSA alone generated predominantly CD4+ T cell responses indicating HLA Class II mediated antigen presentation, while the combination of the antigen plus antibody generated both CD4+ and CD8+ T cell responses. We further demonstrated that the PSA/anti-PSA complexes generated HLA allele-restricted CD8+ T cell responses to peptides known to bind to the HLA-A2 allele. We had previously demonstrated that apoptotic prostate cancer cell lines could induce immune responses when exposed to and presented by DC, however, these studies lacked specificity. However, specificity was demonstrated using tumor cell lysates from the LNCaP cell line which produces PSA. T cells generated by the cell membrane preparations from this cell line were responsive to DC that had been armed with PSA using the antigen/antibody complexes.

One of the confounding issues in immunotherapy is the extent to which HLA allele differences in the population will restrict the capacity of a given individual to generate effective cellular immunity when a specific protein is administered as a vaccine. Using T cell lines and T cell clones generated from a patient with prostate cancer and a patient with granulomatous prostatitis, peptides were identified that were specifically presented by the HLA-B7 molecule. In addition, we also demonstrated that T cell response to PSA that was restricted by an HLA Class II allele DRB*1501. This was the first demonstration of a Class II

restricted response to PSA and extends a number of HLA Class I alleles capable of presenting PSA peptides.

The knowledge gained by these experiments supports the concept that DC can be used to test *de novo* immune responses to a number of different sources of candidate vaccines for prostatic cancer immunotherapy. The primary target was PSA - a protein that is overproduced by prostatic cancer cells relative to that produced by the nonmalignant counterpart. Other candidate sources of antigens that might be used for immunotherapy were also studied. These included cell membrane preparations from prostate cancer cell lines and whole RNA preparations from long-term prostate cancer cell lines. While all preparations acquired by DC generated some T cell responses, the most robust and extensive responses were generated by DC that have acquired PSA in the form of an antigen/antibody complex. This study essentially provides a platform for the introduction of tumor specific antigens to dendritic cells and demonstrates the capacity of this modality to generate immune responses *in vitro*. We intend to extend these studies to evaluate immune responses in individuals with a number of different HLA Class I alleles to determine the extent to which a PSA/anti-PSA antigen/antibody complex could be used directly as an immunogen or delivered by armed dendritic cells for the immunotherapy of prostate cancers.

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"PSA as a candidate cell antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome." S. Ponniah, I. Arah, R. B. Alexander. The Prostate 44: 49-54, 2000

"Generation of CD4+ and CD8+ T lymphocyte responses by dendritic cells armed with PSA/anti-PSA (antigen:antibody) complexes." K.A. Berlyn, B. Schultes, B. Leveugle, A. Noujaim, R.B. Alexander, D.L. Mann. Clinical Immunology 101(3): 276-83, 2001.

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Review. "Therapeutic antibodies for prostate cancer." B. Leveugle, D. Mann, R. Madiyalkan, A. Noujaim.

PERSONNEL SUPPORT BY THIS AWARD

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UM4081R2
12/21/01

Generation of CD4+ and CD8+ T Lymphocyte Responses by Dendritic Cells Armed with PSA/anti-PSA (antigen:antibody) Complexes

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Running title: T cell response to PSA immune complex armed dendritic cells

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ABSTRACT

Dendritic cells (DC) acquire antigens through a number of cell surface structures including receptors for the Fc portion of immunoglobulins and mannose. Little is known about the effects of the antigen uptake via these receptors on antigen processing and presentation. We compared the capacity of DC to generate CD4+ and CD8+ T cell responses after exposure to prostate specific antigen (PSA) alone, PSA targeted to the mannose receptor (mannosylated PSA(PSA-m)) or to Fc receptors by combining PSA with an anti-PSA-antibody (AR47.47). Autologous CD3+ T cells were added to monocyte-derived immature DC that had been cultured with GM-CSF/IL-4 for 4 days, exposed to antigen, matured with CD40L or TNFα/IFNα. After several rounds of stimulation, T cell responses were assessed by intracellular IFNγ production using flow cytometry. Both CD4+ and CD8+ T cell responses were observed after stimulation with DC exposed to the PSA/anti-PSA complexes whereas CD4+ predominated over CD8+ T cell responses after stimulation with PSA armed DC or PSA-m. These CD8+ T cells responded when re-challenged with DC pulsed with HLA allele-restricted PSA peptides. These results indicate that PSA and PSA-m are processed primarily through pathways that favor HLA Class II presentation, while the PSA/anti-PSA immune complexes are processed through both Class I and Class II pathways in monocyte-derived DC. These findings have potential applications in designing more effective cancer vaccines for prostate cancer.

INTRODUCTION

Prostate cancer is the second most common cause of cancer deaths in American males and the most common solid tumor with nearly 180,000 new cases each year (1-2). Surgical or radiotherapeutic intervention is effective treatment for localized disease, however, micrometastases are not eliminated by these measures. Standard chemotherapy and/or hormonal treatment are not always successful in eradicating residual tumor cells.

In other malignancies, immune-based therapies have achieved some degree of success in cancers that have not responded to conventional treatment and are now also being investigated as an adjunct treatment modality in prostatic cancer (3-9).

Over the past several years, a number of studies document the potential use of dendritic cells (DC) as a delivery vehicle for cancer vaccines (10-12). In these studies, T cell-mediated immune destruction of tumors has been observed in patients who have been vaccinated with DC armed *ex vivo* with "tumor antigens." The DC-based vaccines that appear to be the most effective are those that generate CD8+ cytotoxic T cell (CTL) responses targeted to the tumor cell. These responses are mediated by CTL recognition of the cognate tumor antigen(s) presented in the form of peptides by one or more of the HLA class I alleles on the tumor cell surface. To generate the CTL, the DC must acquire and process the targeted antigen through pathways that generate the same allele-specific peptides that are presented by the tumor cells. DC are capable of inducing primary T lymphocyte immune responses and activate memory T lymphocytes (22) through unique mechanisms. Unlike other APC, DC can acquire antigens from their environment and process them through both HLA Class I and Class II pathways (cross-presentation) allowing engagement of cognate receptors and activation of CD8+ and CD4+ T cells. This feature is important: It is now quite clear that robust CTL responses are dependent on "help" from CD4+ T cells. These cells recognize antigenic peptides presented by HLA Class II molecules on the APC. Upon cognate antigen recognition, help is provided by production of cytokines, particularly those that favor a Th1 type immune response. Thus it is important that the APC process the candidate tumor antigen(s) through pathways that generate epitopes that are capable of being presented by one or more of the HLA Class I and Class II alleles that are represented in the candidates for vaccine therapy. In studies aiming at immunotherapy of prostate cancer, a number of products of the prostate cancer cell might

serve as candidate tumor antigens. However, clinical trials have been initiated mainly with two - prostate specific membrane antigens (PSMA) and PSA (3-9).

PSA is a serine protease produced by prostate epithelium and used as a marker for prostate cancer (13). PSA is a secreted protein found in human serum and can also be identified in the cytoplasmic compartment of prostate epithelium and prostate tumor cells (14). The increasing serum levels of PSA associated with the development of prostate cancer make it both a useful marker for disease and a potential target for immunotherapy. PSA has also been shown to be immunogenic in studies where PBMC have been repeatedly stimulated *in vitro* with the intact protein or HLA allele-restricted PSA peptides (15-19). PSA has also been shown to be targeted by the immune system of some patients with chronic prostatitis/chronic pelvic pain syndrome, evidence that this protein can be immunogenic *in vivo* (20-21).

Dendritic cells (DC) acquire antigens through a number of receptors including receptors for the Fc portion of immunoglobulins, for mannose or complement. Little is known about the effects of the antigen uptake route on antigen processing. We conducted an *in vitro* study to compare the capacity of DC to generate CD4$^+$ and CD8$^+$ T cell responses after processing of PSA alone, or PSA targeted to the mannose receptor (mannosylated PSA) or to Fc receptors (PSA complexed with anti-PSA antibody AR47.47). The objectives of this study were to develop methods that would successfully generate both CD4+ and CD8+ T cell responses to PSA *in vitro*. This was achieved with immature DC exposed to PSA together with a monoclonal PSA-specific antibody (AR47.47). The CD8+ T lymphocytes responding to DC armed with the antigen-antibody combinations recognized 2 different HLA-A*0201 restricted PSA peptides. On the other hand, arming DC with PSA alone or PSA-m preferentially induced CD4+ responses.

MATERIALS AND METHODS

Source of Cells

PBMC were isolated from the apheresis products from normal volunteers by ficoll-hypaque (Sigma, St. Louis, MO) gradient centrifugation, viably frozen using an automated cell freezer (Gordinier Electronics, Roseville, MI) in RPMI (Life Technologies, Frederick, MD) containing 40% human Ab serum (Gemini Bio-

Products, Woodland, CA) and 10% DMSO (Sigma, St. Louis, MO) and stored in the vapor phase of liquid nitrogen until used. DNA was prepared from a portion of the cells and used for molecular HLA typing.

Isolation of DC by negative selection

DC precursors were prepared from freshly-thawed PBMC by negative selection using immunomagnetic bead depletion. PBMC were incubated on ice for 30 min with mouse anti-human CD3, CD16 and CD19 (Caltag, Burlingame, CA). Excess antibody was removed by washing the cells with PBS/0.1% BSA and the cells were incubated with Pan Mouse IgG immunomagnetic beads (Dynal, Lake Success, NY) for 30 min on ice. The tube was placed against a magnet to remove the cell:bead complexes and the supernatant containing the lineage-depleted DC precursors collected.

DC cultures

The lineage-depleted DC precursors were washed, resuspended in cRPMI (RPMI supplemented with 1% glutamine and 10% heat-inactivated human Ab serum) containing GM-CSF (1000 U/ml) and IL-4 (1000 U/ml)(R & D Systems, Minneapolis, MN) and cultured at 37°C in 5% CO_2 at 0.5 x 10^6 cells/well in 24 well plates. On the fourth day of culture, the cells were pulsed with antigen and incubated for an additional 8 h. Several agents known to mature DC were then added to the cultures. These included soluble trimeric CD40L (kindly provided by Immunex, Seattle, WA), TNFα (10 µg/ml) and/or IFNα (50 µ/ml). The matured DC were harvested on the seventh day of culture, analyzed for phenotypic markers by flow cytometry and used in functional studies.

Phenotypic analysis of DC by flow cytometry

DC were analyzed for cell surface marker expression by flow cytometry. Briefly, the cells were aliquotted into polystyrene tubes and stained for surface markers with fluorochrome-labeled murine antibodies. Cell surface markers include: HLA-A,B,C, HLA-DR, CD14, CD11c, CD123, CD4, CD40, CD83, CD86, CD80, CD16, CD32, CD64 (Becton Dickinson, San Jose, CA). Following a 30 min incubation on ice, the cells were washed with PBS and pelleted by centrifugation. The cell pellets were resuspended in 250 µl of fixative (2% paraformaldehyde). The data was acquired using a FACSCan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with Cellquest software (Becton-Dickinson, San Jose, CA).

Isolation of T cells

Responder CD3+ T lymphocytes were isolated from thawed PBMC by negative selection (Dynal, Lake Success, NY). Briefly, the cells were incubated on ice for 30 min with a mixture of antibodies to CD14, CD16, CD56 and HLA Class II DR/DP. Excess antibodies were removed by washing with PBS/0.1% BSA. The cells were incubated for 30 min at room temperature with immunomagnetic beads coated with an anti-mouse IgG antibody (Dynal, Lake Success, NY). The cells were placed against a magnet and the T lymphocytes were isolated from the supernatant.

Preparation of antigens

PSA was purchased from Scripps Laboratories (San Diego, CA) and the murine monoclonal anti-PSA antibody AR47.47 R6R6 (ProstaRex™) was kindly provided by AltaRex Corporation, Edmonton, Alberta, Canada. This IgG1 antibody reacts specifically with an epitope that maps to the region of aa 137-144 (sequence EPEEFLT) of PSA. PSA and AR47.47 were diluted in cRPMI to concentrations of 5 and 25 μg/ml, respectively. When mixed at these concentrations, approximate equimolar amounts of PSA and anti-PSA were achieved. PSA was mannosylated as follows: 100 μg of PSA was combined with 100 μg a-D-mannopyranosylphenylisothiocyanate (a-D-M) and 2 μl N-methylmorpholine in 460 μl of PBS and stirred overnight at room temperature. Excess a-D-M was hydrolyzed by the addition of 100 μl of 1M Trizma-base (pH 9.5). Unconjugated mannose residues were removed by dialysis against PBS and stored at 4°C. Two HLA-A2 specific PSA peptides (pep1, FLTPKKLQCV; pep2, KLQCVDLHV) and an HIV-1 peptide (SYNTVAVL) were synthesized by the Biopolymer Laboratory, University of Maryland, Baltimore, MD, diluted in cRPMI and added to the DC preparations at concentrations of 5 μg/ml.

Antigen stimulation assays

T lymphocytes were plated in twenty-four well plates at a concentration of 1 x 10^6 cells/well and to which were added 5 x 10^4 DC that were antigen naive or that had been exposed to PSA, PSA-m, the anti-PSA monoclonal antibody or the combination of PSA and the monoclonal antibody. At day 7 the T cells were harvested, washed and cultured for an additional 7 days with DC that had been armed as described above in cRPMI supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml)(R&D Systems, Minneapolis, MN). T cells

were restimulated for 24 h with armed DC (in combinations described in the Results) and responses assessed by measuring intracellular cytokine production in CD4+ and CD8+ T lymphocytes.

Monoclonal antibody (AR47.47) and AR47.47-PSA immune complex binding to DC

The anti-PSA monoclonal antibody AR47.47 was labeled with Fluorescein-EX (Molecular Probes, Eugene, OR) following manufacturers instructions. Equimolar concentrations of PSA were mixed with 1.25 μg/ml, 2.5 μg/ml, 5.0 μg/ml, and 25 μg/ml of AR47.47-FITC and added to freshly isolated DC precursors, to DC that had been cultured for 4 days in the presence of GM-CSF/ IL-4 and to DC that had been matured. The antibody-PSA combination was incubated for 1 h at 37°C, washed, pelleted, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. DC were gated in the sideward/forward scatter based on DC preparations stained in parallel with the complete DC marker panel (see "Phenotypic Analysis of DC by Flow Cytometry").

Detection of intracellular cytokine expression by flow cytometry

Intracellular cytokine production by CD4+ and CD8+ T cells was measured by flow cytometry. Brefeldin A (10 μg/ml)(Pharmingen, San Diego, CA) was added to the T cell cultures 2 h after restimulation with antigen armed DC. After an additional 18 h of culture, cells were incubated with staining buffer (PBS with 1% human Ab serum) for 15 min at 4°C, washed again, pelleted and fluorochrome labeled antibodies to CD3, CD4, or CD8 (Becton Dickinson, San Jose, CA) added. The cells were fixed and permeabilized by incubation with perm/fix solution (Pharmingen, San Diego, CA) for 20 min on ice, washed and antibodies to IFNγ or appropriate isotype controls (Pharmingen, San Diego, CA) added. After incubation for 30 min on ice, the cells were washed, resuspended in staining buffer containing 2% paraformaldehyde and analyzed by flow cytometry.

RESULTS

AR47.47 Antibody and AR47.47-PSA Immune Complex Binding to DC at Different Stages of Development and Maturation

DC capture antigens through interactions with a number of cell surface structures including the Fcγ type I, II, and type III receptors (CD16, CD32, CD64, (Table I)). The percent of DC expressing these receptors differed depending on their stage of maturation/differentiation. The number of DC expressing CD16 was highest after culture for 4 days with GM-CSF/IL-4 and was nearly absent on DC after maturation. CD32 and CD64 were found on the majority of DC precursors. CD32 was expressed on upwards of 90% of immature DC and declined to about 40% on mature DC. The number of CD64 expressing cells declined as the DC were differentiated and matured. To determine if the anti-PSA monoclonal antibody AR47.47 and AR47.47-PSA immune complexes would bind to DC and if the stage of DC differentiation and maturation affected binding, a range of concentrations of the fluorescein-labeled AR47.47 as well as AR47.47-PSA-FITC-complexes were added to three different populations of DC: I) freshly isolated myloid DC precursors (surface phenotype-CD11c+, HLA-DR+, CD14+), II) DC after culture for 4 days in GM-CSF/IL-4 (surface phenotype-CD11c+, HLA-DR+, CD14-, CD86+) and III) DC that had been further matured by culturing for an additional 3 days with TNFα and IFNα (surface phenotype-CD11c+, HLA-DR+, CD86+, CD40+, CD80+, CD83+). The percentage of cells that reacted with AR47.47 and AR47.47-PSA complexes were calculated based on binding to CD11c+, HLA-DR+ cells (Table II). At the concentrations tested, AR47.47 bound to a low percentage of freshly isolated DC precursors. The number of cells binding the antibody increased after culture for 4 days in GM-CSF and IL-4. Interestingly, the number of cells binding AR47.47-FITC was significantly higher in the presence of PSA, indicating that the Fc receptors have enhanced binding to immune complexed antibody over the antibody alone, or that immune complexes bind to different and/or more abundant receptors on DC precursors and immature DC. No binding of the AR47.47 or the immune complexes occurred to mature DC.

T lymphocyte responses to DC armed with PSA or PSA/anti-PSA

DC precursors isolated from PBMC and cultured for 4 days in GM-CSF/IL-4 were exposed to PSA, the anti-PSA monoclonal antibody and the antibody-PSA combination. After maturation, armed or unarmed DC were co-cultured with autologous T-cells, restimulated with armed and un-armed DC and the number of

CD4+ and CD8+ cells producing IFNγ determined. The results of a representative experiment are shown in Figure 1. Little to no difference in number of IFNγ producing CD4+ or CD8+ T cells were found in co-cultures with DC that were not exposed to antigen (negative control) or T cells cultured with DC that had been exposed to the monoclonal antibody and were then restimulated with armed or un-armed DC. PSA armed DC stimulated CD4+ T cell responses that were consistently greater than CD8+ T cell responses. However, CD8+ T cell responses were observed on restimulation with immune complex armed DC. When T cells were cultured with antigen-antibody armed DC and restimulated with PSA armed DC, CD4+ T cell responses were substantially greater than CD8+ responses. On the other hand, consistent CD4+ and CD8+ IFNγ responses were only generated in T cells exposed to and restimulated by antigen-antibody armed DC. Since IFNγ T cell responses were not generated to the AR47.47 antibody, it is expected that the response to the complex is directed at the PSA. The increase in CD8+ T cell responses to PSA presented in combination with the antibody compared to the responses with free PSA alone indicate that the immune complex enhances antigen processing through the HLA Class I pathways.

PSA/anti-PSA complex-armed DC generate HLA-A*0201 peptide-specific T lymphocyte responses

We compared the ability of PSA/anti-PSA immune complexes and PSA armed DC to generate T lymphocyte responses to two PSA peptides that are known to be restricted by the HLA-A*0201 allele (Figure 2). DC were prepared as described and exposed to PSA or PSA/anti-PSA after 4 days of culture in GM-CSF/IL-4. Following maturation with CD40L or TNFα and IFNα, the armed DC were cultured with autologous CD3+ selected T lymphocytes for 7 days. The cells were harvested, newly armed DC added and the cells cultured for an additional 7 days in media supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). At day 14, the cells were harvested, restimulated with DC that had been armed with PSA or PSA/anti-PSA at day 4 and matured for 3 days or with matured DC that were pulsed with the two PSA-derived peptides and analyzed for IFNγ release. Results of a representative experiment (1 of 3) are shown in Figure 2. In T cells cultured with PSA-armed DC for 2 wk, low levels of CD4+ responses were detected upon restimulation with the PSA-derived peptides. Peptide 2 (KLQCVDLHV) also induced small CD8+ responses. Consistent with the previous results, restimulation of T cells with PSA-armed DC resulted in CD4+ T cell responses, and low

levels of responding CD8+ T cells. Restimulation with the PSA/anti-PSA complex showed similar activation of CD4+ as well as CD8+ INFγ responses. T cells cultured with PSA/anti-PSA armed DC for 2 wk were able to respond to both PSA-derived peptides. Peptide-restricted responses were approximately twice as strong in T cells cultured with PSA/anti-PSA armed DC before restimulation compared to the responses to PSA-armed DC. It is interesting to note that T cells propagated in the presence of PSA-armed DC are able to respond weakly to peptide 2 (KLQCVDLHV), but do not release IFNγ by CD8+ T cells upon restimulation with PSA-armed DC. This suggests the possibility that this peptide may be presented by HLA Class II molecules as well as by Class I, but that the generation of T cells specific for this peptide are not effectively generated by DC loaded with PSA. No responses were found when the T cells that had been cultured with immune complex were stimulated with mature DC that had been pulsed with an HLA-A*0201 restricted HIV-1 gag peptide (data not shown).

T cell responses to DC armed with PSA or PSA-m

DC express receptors that bind mannose and related carbohydrates. Experiments were conducted to compare the T cell responses to DC that had been armed with PSA or PSA that had been conjugated to mannose. T cells were exposed through two weekly rounds of stimulation to DC armed with PSA or PSA-m and CD4+ and CD8+ IFNγ responses measured after re-exposure to armed DC (Table III). In three separate experiments, CD4+ T cell responses predominated within the activated cell population and were consistently higher when DC were loaded with PSA-m compared to responses to PSA armed DC. Small to modest increases (over controls) in number of activated CD8+ T cells were observed. However, the CD8+ T cell response to PSA-m was far below responses seen with AR47.47-PSA immune complexes.

Discussion

A number of studies indicate that successful tumor vaccines possess epitopes that can be presented by HLA Class I and Class II molecules so that CD8+ and CD4+ T cell responses are generated. The capacity to readily isolate DC precursors from peripheral blood and to develop these cells into primary APC *in vitro*

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offers the possibility to identify effective antigen candidates for immunotherapy and determine ways of introducing antigens to DC in order to effect both CD4+ and CD8+ T cell responses.

Current obstacles in generating effective DC for vaccine trials include poor presentation of antigens on either MHC class I or class II molecules, particularly if the antigen needs to be processed from extracellular sources. Antigens delivered to the extracellular environment of DC may often generate potent CD4+ T cells responses but poor CTL, as confirmed here in experiments using DC loaded with PSA or mannosylated PSA. On the other hand, proteins produced in or delivered to the cytosolic compartment of DC are not presented efficiently on MHC class II molecules. Such approaches can generate CTL responses but lack professional T cell help to maintain CTL *in vivo*. Here we show that both CD8+ and CD4+ T cell responses were generated by DC armed with an immune complex containing the xenogenic anti-PSA antibody AR47.47 but that only CD4+ T cell responses were generated by DC armed with PSA alone.

DC derived from myeloid precursors in peripheral blood and cultured with GM-CSF and IL-4 express high and low affinity receptors for the Fc portion of the immunoglobulin molecule. These receptors have been shown to mediate antigen acquisition by DC when presented together with an antibody as an immune complex (23-27). Coccia and Brams (1998) found higher antibody titers in hu-PBL-SCID mice immunized with DC pulsed *ex vivo* with PSA-antibody complexes compared to mice immunized with DC pulsed with PSA alone (28). Our *in vitro* studies also indicate that DC armed with PSA in the form of immune complexes have a greater capacity to generate Th1 type immune responses compared to DC that have been armed with PSA as CD8+ responses were consistently generated. These data indicate that antigens acquired by DC via the Fc receptor enhance cross-presentation and processing through the Class I pathway. This contention is supported by studies in mice demonstrating that Fc gamma receptor mediated antigen acquisition by immature DC promoted MHC Class I presentation (23).

DC acquire antigenic substances through macropinocytosis or receptor mediated endocytosis (22). A number of these receptors have been identified and have been specifically targeted as means of enhancing antigen uptake. The mannose receptor is a C-type lectin receptor expressed on DC. We compared the capacity of DC exposed to PSA or PSA-m to generate CD4+ and CD8+ T cell responses. In our experiments, CD4+ T cell responses predominated when CD3+ T cells were exposed to PSA armed DC and the number of IFNγ

producing CD4+ T cells were consistently higher when T cells were exposed to DC armed with PSA-m. However, CD8+ T cell responses were consistently low in cultures with PSA or PSA-m armed DC. Thus targeting the mannose receptor did not greatly enhance cross-presentation of PSA.

We considered the possibility that the stage of DC maturation might influence Class I and Class II presentation and thus the differences in the CD8+ and/or CD4+ T cell responses that were observed. No responses were observed when PSA or the PSA immune complexes were added to freshly isolated DC precursors prior to culture with GM-CSF and IL-4 or when the antigens were added after maturation (data not shown). We also found that the T cell responses occurred only when peptides were added to DC after full maturation. These differences in antigen presentation *in vitro* (and probably *in vivo*) undoubtedly depend on the relative abundance and/or differences in the cell surface structures that are known to be expressed on DC and that change during maturation into potent APC. The monoclonal antibody used in this study was found to bind to DC precursors and immature DC but not to mature DC, correlating with the expression of CD16, CD32 and CD64. These results are consistent with those of our functional studies where T cell responses were generated with DC armed with the immune complexes after the initial culture period but not by DC that were exposed to the antigens after maturation. Flow cytometry studies also showed that the directly labeled AR47.47 antibody bound significantly better to DC precursors and immature DC when combined with PSA indicating that the antibody alone and the antibody-antigen complex may be taken up by different Fc receptors. Further studies on the receptors targeted are currently underway in our laboratory.

The ideal antigen for cancer immunotherapy would be one that is produced exclusively by the malignant cell of a particular histological type and contains sequences that can be processed to epitopes that can be presented by a wide range of HLA Class I and Class II alleles. This ideal situation in all probability does not exist so investigators have searched for and identified epitopes in products of cancer cells that appear to be immunodominant and are restricted by the most frequent HLA alleles in the population. In prostate cancer the antigens most frequently studied have been PSA and PSMA. A number of different groups have identified sequences in these proteins that are HLA allele restricted targets for cytotoxic T cells (15-17). The majority of these studies have been directed at identifying the epitopes that are presented by HLA-A2, the most frequent Class I allele in the North American-European population. Several HLA-A2 restricted CTL

epitopes have been identified in PSA (15-17), two of which were used in our study to show that the CD8+ T cell responses generated with PSA and/or PSA immune complex armed DC were HLA allele restricted. Similar results were obtained with more than one HLA-A*0201 donor.

To date the factors that are required for or that foster cross-presentation are unknown. In our studies, HLA Class II mediated CD4+ T cell responses were mediated by DC that acquired PSA through unknown receptors. This same type of response predominated with PSA targeted to the mannose receptor. On the other hand, DC that incorporated PSA in the form of immune complexes generated both CD4+ and CD8+ T cell response indicating that Fc receptor mediated antigen acquisition enhances cross presentation. These results suggest the possibility that effective cancer vaccines may be generated by DC exposed *in vitro* to candidate "cancer antigens" combined with antibodies prior to administration. Alternatively, these immune complexes may be effective in generating potent CD4+ helper cells and CTL by targeting DC *in vivo*. Our observation may explain the activation of antigen specific B and T cell responses in patients in clinical trials treated with two low dose antibodies that target CA125 (OvaRex® MAb-B43.13) and Muc-1 (BrevaRex® MAb-AR 20.5) (29-31).

We have also observed T cell responses with DC armed with PSA or the PSA immune complex using PBMC from donors with other HLA Class I alleles and are in the process of identifying the PSA peptide-allele restriction. CD4+ T cell responses to PSA have always been observed in our studies, however, the number of different HLA Class II alleles was limited to four. One allele that was common to two of the cells was DRB1*0401 that has previously been shown to present a PSA peptide (17). We have tentatively identified a PSA response restricted to DRB1*1501 (unpublished data).

In summary, our results extend the observations of other investigators showing the efficacy of delivering antigens as immune complexes to DC in order to generate both HLA Class I and Class II mediated T cell responses in circumstances where the response may be limited to the Class II CD4+ T cell recognition of an antigen acquired by DC exposed to the antigen alone.

TABLE I

Distribution of Fcγ Receptors on DC at Different Stages of Maturation

Fcγ Receptor	Days in culture		
	0	4	7
	Positive Cells [%] (gated on DC)		
CD16 (FcγRIII)	3 - 15	40 - 50	0.5 - 5
CD32 (FcγRII)	89 - 98	70 - 95	40 - 45
CD64 (FcγRI)	92 - 99	28 - 60	4 - 10

DC isolated from PBMC as described and tested for Fcγ receptor expression with fluorescence labeled antibodies at day 0, after 4 days in culture with GM-CSF/IL-4 and after maturation with TNFα and IFNα (7 days). Binding was determined on CD11c+, HLA-DR+ cells and is expressed as percentage of positive cells within the gate. Numbers are the ranges obtained in 7 experiments.

TABLE II

PSA-Specific Monoclonal Antibody AR47.47 and AR47.47-PSA Immune Complex Binding to DC at Different Stages of Maturation

		Days in culture		
		<u>0</u>	<u>4</u>	<u>7</u>
	Antibody Concentration	Positive Cells (gated on DC) [%]		
AR47.47-FITC	1.25 μg/ml	0.13	0.68	
	2.5 μg/ml	0.16	1.56	
	5 μg/ml	1.84	9.95	1.19
	25 μg/ml	7.33	28.10	
AR47.47-FITC + PSA	1.25 μg/ml	1.82	2.41	
	2.5 μg/ml	3.91	8.70	
	5 μg/ml	5.38	23.40	1.56
	25 μg/ml	18.00	72.11	

DC isolated from PBMC as described and tested for antibody binding at day 0, after 4 days in culture with GM-CSF/IL-4 and after maturation with TNFα + IFNα (7 days). Binding was determined on CD11c+, HLA-DR+ cells and is expressed as percentage of positive cells within the gate.

TABLE III

Comparison of T Cell Responses to Dendritic Cells Armed with PSA or PSA-m

Primary stimulation	Restimulation	IFNγ+ cells/10^5 CD4+	CD8+
O	PSA	33	42
PSA	O	109	41
PSA	PSA	438	88
PSA	PSA-m	566	92
O	PSA-m	80	61
PSA-m	O	111	82
PSA-m	PSA-m	894	122
PSA-m	PSA	504	113

T cells were exposed to DC armed with PSA or PSA-m and cultured as described in methods. At day 14, T cells were restimulated with unarmed or antigen armed DC and numbers of CD4+ and CD8+ T cells producing IFNγ determined by flow cytometry.

Figure 1. In vitro T-cell activation by monocyte-derived dendritic cells
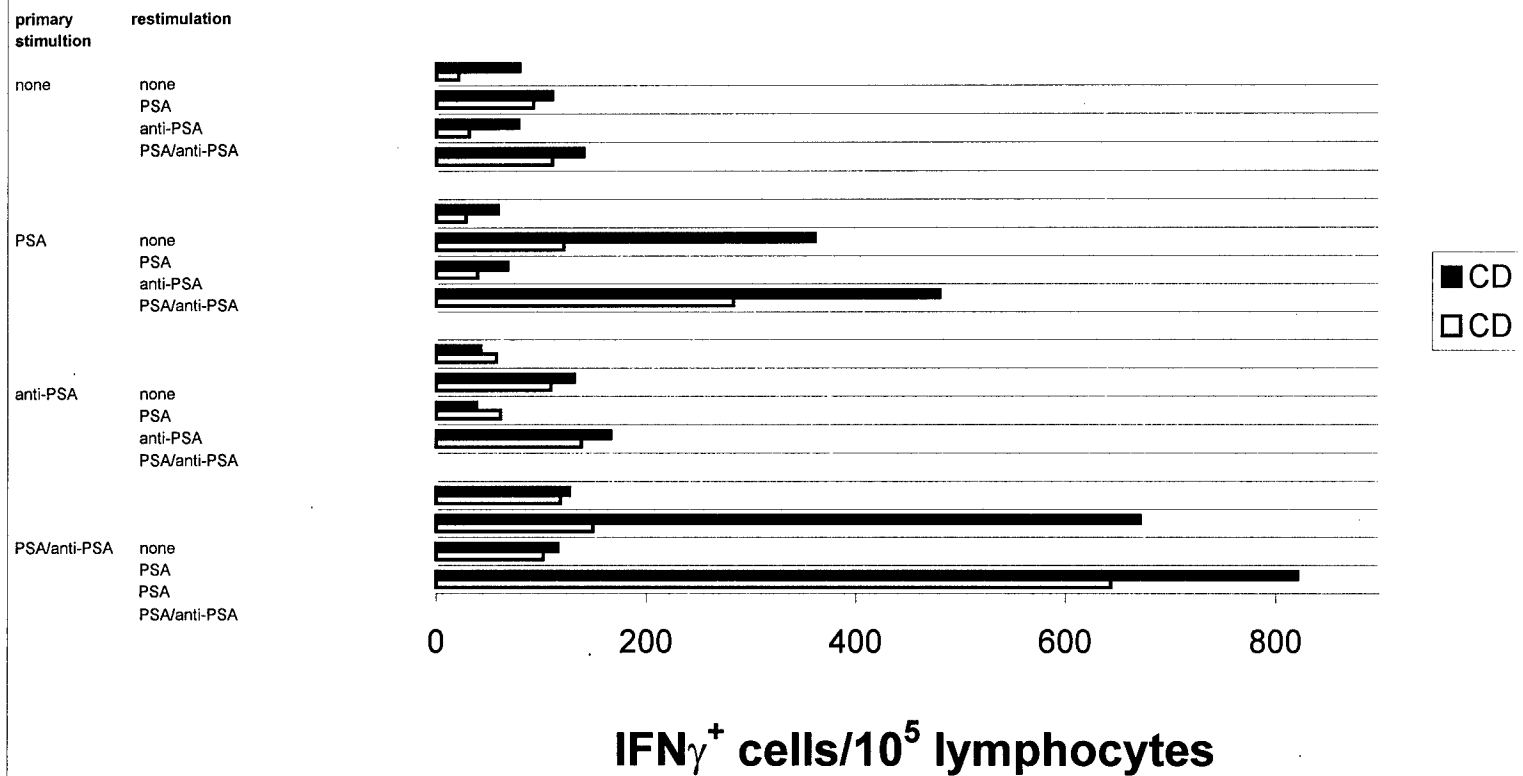
Figure 1. CD4+ and CD8+ T cell responses generated by DC armed with PSA or with PSA/anti-PSA immune complex. CD3+ T cells were cultured with DC prepared from PBMC and armed with PSA or with PSA combined with a monoclonal PSA antibody as described in Methods. After two rounds of weekly stimulation, the T cells were restimulated with unarmed DC or armed with antigens as indicated. Responses measured as numbers of CD4+ and CD8+ T cells producing intracellular IFNγ.

Figure 2. T-cell response to PSA peptides generated by DC armed with PSA or PSA/anti-PSA complex
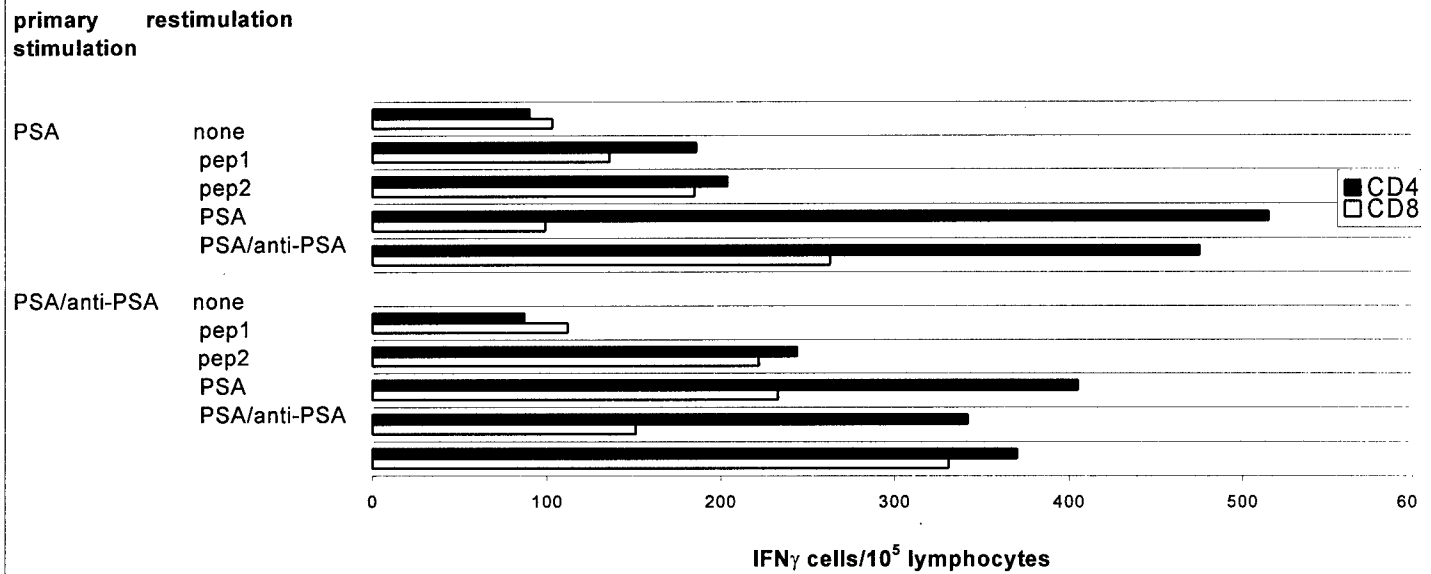
Figure 2. CD8+ T cell responses generated by DC armed with PSA combined with an anti-PSA monoclonal antibody recognize HLA-A*0201 restricted PSA peptides. CD3+ T cells were cultured with DC armed with PSA or with PSA/anti-PSA combination as described in Methods. After two rounds of weekly stimuli, the T cells were restimulated with DC armed with PSA, PSA/anti-PSA and two HLA-A*0201 allele restricted peptides. Responses were measured as numbers of CD4+ and CD8+ T cells producing IFNγ.

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PSA Is a Candidate Self-Antigen in Autoimmune Chronic Prostatitis/Chronic Pelvic Pain Syndrome

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BACKGROUND. Previous studies demonstrated that recognition of seminal plasma antigens can occur in patients with chronic prostatitis/chronic pelvic pain syndrome. This suggests that an autoimmune component may contribute to symptoms in some men. To determine if any of the principal secretory proteins of the prostate could be candidate antigens in auto-immune prostatitis, we examined the recall proliferative response of purified CD4 T cells in patients with chronic prostatitis and in normal volunteers using purified seminal plasma antigens and autologous dendritic cells.

METHODS. Peripheral blood mononuclear cells were harvested from 14 patients with chronic prostatitis and 12 normal volunteers by density gradient centrifugation. The stimulating cells were irradiated autologous dendritic cells produced by culture of monocyte-enriched fractions with IL-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). Purified CD4 T cells were the responding population. Recall proliferation assays were performed, using purified seminal plasma proteins as antigens.

RESULTS. In 14 patients with chronic prostatitis, we detected a greater than 2-fold increase in proliferative response to PSA compared to control in 5 patients (36%). No response to Prostatic Acid Phosphatase (PAP) or β-microseminoprotein was observed in these 14 patients. In 12 normal volunteer donors with no history of genitourinary disease or symptoms, no proliferative response above background was observed for any prostatic antigen.

CONCLUSIONS. The data suggest that some men with symptoms of chronic prostatitis have evidence of a proliferative CD4 T-cell response to PSA. PSA is a candidate antigen in chronic prostatitis/chronic pelvic pain syndrome and may be an appropriate target for immuno-therapy for prostatic cancer. *Prostate 44:49–54, 2000.*   © 2000 Wiley-Liss, Inc.

KEY WORDS: prostatitis; prostate-specific antigen; autoimmunity; T lymphocytes; dendritic cells; prostate cancer

INTRODUCTION

Chronic prostatitis/chronic pelvic pain syndrome is a common diagnosis, but very little is understood about the etiology of the disease. Men with this syndrome present with an episodic and relapsing condition characterized principally by pain in the pelvic region, voiding symptoms, and effects on sexual function [1]. These symptoms cannot be distinguished from those of men with acute bacterial infections of the prostate gland; however, the overwhelming majority of men with chronic symptoms cannot be demonstrated to have bacterial infection [2]. The disease represents a major problem in the US, resulting in 2 million office visits yearly to primary care physicians and urologists [3]. Most of these men are treated with prolonged courses of antimicrobials, with unknown and doubtful benefit.

We recently reported that some men with chronic prostatitis/chronic pelvic pain syndrome have evidence of CD4 T lymphocyte recognition of seminal plasma, derived both from normal men and men with seminal vesicle atresia [4]. We used seminal plasma as the source of antigens in that study because a significant proportion of the volume of the semen is contributed by the prostate. In addition, semen from men with seminal vesicle atresia consists almost entirely of the secretions of the prostate and is characterized by azoospermia, absent fructose, and low volume. These data suggested that some men with chronic prostatitis/chronic pelvic pain syndrome could have an autoimmune component to their disease that could be either a cause or consequence of their symptoms.

To further define the potential antigens contained within the seminal plasma that could be recognized in these patients, we studied patients and normals using purified seminal plasma proteins of prostatic origin. The major secretory proteins of the prostate contained within the seminal plasma are prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and β-microseminoprotein (β-MSP) [5]. These proteins, purified from human seminal plasma, were used as antigens in CD4 T-cell recall proliferation assays in patients with chronic prostatitis/chronic pelvic pain syndrome and normal volunteers. We found that some men with chronic prostatitis/chronic pelvic pain syndrome had a CD4 T-cell proliferative response to PSA and that this was not observed in normal volunteers. This suggests that PSA is a candidate antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome.

MATERIALS AND METHODS

Culture Medium

Culture medium consisted of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% human AB serum (heat-inactivated) (Gemini Bioproducts, Calabasas, CA), 2% L-glutamine, and penicillin/streptomycin (Biofluids, Rockville, MD).

Preparation of Cell Populations

Fifty milliliters of peripheral blood were drawn from each normal volunteer and prostatitis patient into a syringe containing 1,000 units of heparin. The blood samples were centrifuged over a density gradient of Lymphocyte Separation Medium (ICN Biomedicals/Cappel, Aurora, OH) to obtain the peripheral blood mononuclear cell (PBMC) population. The PBMCs were then washed, and resuspended at 5×10^6 cells per ml in a 1:2 dilution of culture medium and

PBS. The cells were then centrifuged over a 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient made up in PBS containing 5% human AB serum at $1,000g$ for 25 min at 4°C. This resulted in the separation of the PBMC into two distinct fractions, a cell population at the 40% Percoll gradient interface and a cell pellet at the bottom of the tube. Upon recovery and washing, the two populations appeared to be well-fractionated/separated on the basis of size of the cells, whereby the interface consisted predominantly of large-sized cells (monocytes and macrophages), while the pellet consisted mainly of small, uniform-sized cells (lymphocytes). The cell population from the interface was then incubated with anti-CD2- and anti-CD19-coated Dynabeads (DYNAL, Inc., Lake Success, NY), followed by depletion using the DYNAL-MPC-1 magnet as per the manufacturer's instructions. This technique always resulted in about 80% of the recovered cells postdepletion being positive for the monocyte surface antigen CD14, with less than 2% each of CD3 cells (T lymphocytes) and CD19 cells (B lymphocytes) as determined by FACS analysis. Similarly, the cell population from the pellet fraction was incubated with a cocktail of anti-CD8-, anti-CD14-, and anti-CD19-coated Dynabeads and subjected to the DYNAL magnet depletion technique. The resulting cell population recovered from the pellet fraction postdepletion was always greater than 90% positive for the T lymphocyte antigen CD4, as determined by FACS analysis.

Dendritic Cell Cultures

The highly enriched CD14 monocyte cell population from each patient was cultured in the presence of the cytokines GM-CSF and IL4 (Genzyme Corporation, Cambridge, MA) at a concentration of 10,000 U/ml for each cytokine. The cells were cultured in 6-well plates at $2–3 \times 10^6$ cells per well in 3 ml of Culture Medium (CM). These were incubated at 37°C and 5% CO_2 for a period of 7–8 days. FACS analysis of the cells harvested at the end of the culture period indicated them to be enriched for cells displaying antigens consistent with a dendritic cell (DC) phenotype. These cells were highly positive for the expression of HLA DR, CD80, and CD86, with low expression of CD83, and negative for the expression of CD14 and CD19 antigens.

Proliferation Assay

Recall antigen proliferation assays were performed using irradiated DC (3,000 cGy, ^{137}Cs source) at 1×10^4 cells/well in the absence and presence of 1×10^5 CD4 T cells/well in 96-well U-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ). Purified prepara-

tions of the prostatic proteins PAP, PSA, and β-micro-seminoprotein PSP94 (Fitzgerald Industries, Inc., Concord, MA) were added to the respective wells at 10 μg/ml, and tetanus toxoid was used at 1:100 dilution of the commercially available product (Connaught Laboratories, Willowdale, Ontario, Canada). Some wells did not have any antigens added to them in order to measure the nonspecific or background stimulation of the CD4 T cells by DC and components of the culture medium. All stimulations were performed in duplicate or triplicate, and the plate was cultured for a period of 5 days at 37°C and 5% CO_2. ^{3}H-thymidine was added at 1 μCi/well on the fourth day of culture, and the plates were harvested on day 5 using a Tomtec cell harvester (Wallac, Inc., Gaithersburg, MD); the counts per minute (CPM) were determined by liquid scintillation, counting with the Beta-plate System (Wallac, Inc.). The stimulation index (SI) was calculated as the mean CPM obtained in the presence of antigen divided by the mean CPM obtained in the absence of antigen for all samples.

Statistical Analysis

The stimulation index was calculated for each antigen in prostatitis patients and normal volunteers. A stimulation index greater then 2, meaning that the proliferative response to the three prostatic antigens was greater than twice the CPM response in the absence of antigen, was interpreted as a significant response for that prostatic antigen. The number of individuals with a significant SI response to each prostatic antigen was compared between the normal and prostatitis groups, using Fisher's exact test.

For the response to the control antigen tetanus toxoid (TT), we expected that most individuals would be responsive to this antigen but that the degree of response would be variable, depending on time since last vaccination, Major Histocompatibility Complex (MHC) haplotype, and other variables. Since patients and volunteers were expected to be responsive to TT, we compared the mean proliferative response of the normal and prostatitis groups using the Wilcoxin rank sum test. A P value less than 0.05 was interpreted as excluding the null hypothesis for both tests.

RESULTS

The characteristics of the patients and volunteers are shown in Table I. The median age of the patients was 41 years (range, 24–57), and the median age of the normal volunteers was 34 years (range, 22–65).

The results of the recall proliferation assays using prostatic antigens are shown in Figure 1. In 5 of the 14 prostatitis patients, a proliferation index greater than 2

TABLE I. Demographics of Prostatitis Patients and Normal Volunteers

Subject number	Age	Race
Prostatitis patients		
Pr85	56	White
Pr86	57	White
Pr87	32	Hispanic
Pr88	54	White
Pr90	49	White
Pr92	24	Black
Pr94	34	White
Pr98	25	White
Pr101	42	White
Pr102	33	White
Pr103	43	White
Pr104	57	White
Pr105	41	White
Pr106	36	White
Normal volunteers		
ND12	39	Asian
ND13	40	Black
ND14	28	White
ND15	38	White
ND16	65	White
ND17	24	Asian
ND18	22	White
ND20	23	White
ND21	26	White
ND22	31	White
ND23	58	White
ND24	42	White

Fig. 1. Purified CD4 T lymphocytes from normal volunteers (left) or prostatitis patients (right) were stimulated with irradiated autologous dendritic cells pulsed with purified prostatic antigens, as shown. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a 5-day coculture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by the CPM in the absence of antigen. PAP, prostatic acid phosphatase; PSA, prostate-specific antigen; bMSP, beta-microseminoprotein.

was observed when PSA was used as the antigen. No response was observed among prostatitis patients to PAP or β-MSP. The stimulation index for normal volunteers did not exceed 2 for any of the three prostatic
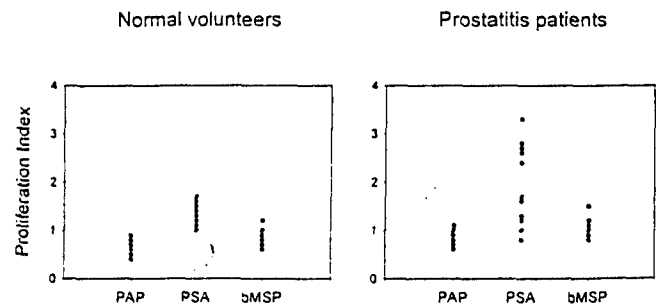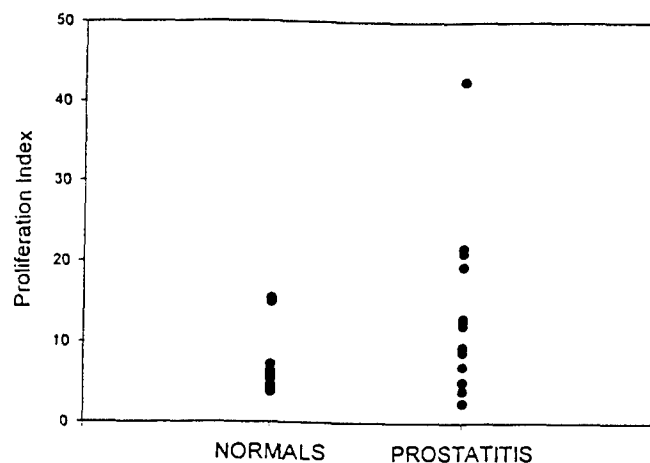
Fig. 2. Purified CD4 T lymphocytes from normal volunteers or prostatitis patients were stimulated with irradiated autologous dendritic cells pulsed with tetanus toxoid. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a 5-day coculture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by the CPM in the absence of antigen.

protein antigens tested. The difference between the response to PSA of prostatitis patients was significantly different from that of normal volunteers ($P = 0.03$, Fisher's exact test). There was no difference between the stimulation indices for PAP or β-MSP when comparing normals to prostatitis patients.

CD4 lymphocytes from normal volunteers and prostatitis patients were responsive to the recall antigen TT, as shown in Figure 2. The mean (SEM) proliferation index was 7.0 (4.0) for normals and 13.5 (10.3) for prostatitis patients. Hence, CD4 T lymphocytes from both groups had comparable positive proliferative responses to TT.

DISCUSSION

We previously demonstrated that men with chronic prostatitis/chronic pelvic pain syndrome have evidence of CD4 T-cell reactivity with seminal plasma and that the antigen being recognized is derived from the prostate [4]. To determine if any of the principal secretory products of the prostate that are secreted into the seminal plasma might be recognized by T cells, we examined CD4 T lymphocytes from men with chronic prostatitis/chronic pelvic pain syndrome for a proliferative response to purified prostatic proteins obtained from the seminal plasma. We found that CD4 T cells from some men with chronic prostatitis/chronic pelvic pain syndrome manifested a proliferative response to PSA that was not present in normal male volunteers. This suggests that PSA is being recognized by the immune system in some men with chronic prostatitis/chronic pelvic pain syndrome, pro-

viding evidence that this disorder represents an autoimmune disease in some patients.

The cause of chronic prostatitis/chronic pelvic pain syndrome is unknown. Infection has long been viewed as the etiology for this problem. Certainly patients with bacterial infection of the prostate do exist. Patients with bacterial infections of the prostate typically respond promptly to therapy with antimicrobial agents. However, most patients with chronic symptoms fail to achieve a durable and lasting remission of symptoms with antimicrobial therapy. In addition, a large body of literature to date has failed to provide convincing evidence that some fastidious organism is responsible for symptoms in a significant proportion of patients. Alternate explanations for chronic prostatitis deserve further study.

The hypothesis that chronic prostatitis/chronic pelvic pain syndrome represents an autoimmune disease in some patients is supported by several observations. First, the chronic, relapsing, and episodic nature of symptoms is consistent with an autoimmune etiology. Second, the prostate is commonly found to contain inflammatory infiltrates when prostate tissue is removed for any reason, typically for prostatic cancer or benign prostatic hypertrophy [6]. The reasons for this inflammation and the implications of its presence are completely unknown. Third, we presented evidence that CD4 T lymphocytes from some men with chronic prostatitis/chronic pelvic pain syndrome manifest a recall proliferative response to seminal plasma [4]. Lastly, we have also shown that the proinflammatory cytokines TNFα and IL-1β are elevated in the semen of some men with chronic prostatitis/chronic pelvic pain syndrome, but not in asymptomatic normal men [7]. These proximal, proinflammatory cytokines are also elevated in the joint fluid in rheumatoid arthritis patients, and the inhibition of these cytokines in patients with rheumatoid arthritis has clearly improved symptoms [8]. These data are consistent with but do not prove that prostatitis is an autoimmune or autoinflammatory condition in some men.

The hallmark of autoimmunity is the demonstration of an immune response against a normal self-antigen. In our study, by choosing to measure the proliferation of T lymphocytes as an indicator of the presence of an immune response in subjects, we were able to demonstrate immunity to PSA in only 5 of the prostatitis patients. It is possible that the use of or inclusion of other immunological assays, such as the detection and measurement of certain cytokines in the lymphocyte cultures stimulated with prostatic antigens, may increase our ability to find evidence of an immune response in a larger number of these patients. Alternatively, the possibility exists that the remaining 9 patients whose cells did not register a proliferative

response may truly have a different etiology for their condition. This is a strong possibility, since the rather loose characterization of pelvic pain as a defining and prerequisite symptom for being considered a patient with prostatitis tends to result in the inclusion of a broad group of individuals who may have other factors contributing to their condition.

The study of antigens in the prostate that could be the target of a T lymphocyte response has principally occurred as a result of investigations in prostate cancer immunotherapy. The central question of cancer immunotherapy is whether antigens exist in human cancers that can be recognized by the immune system, and whether this recognition can be therapeutic in patients with cancer. The description of many such tumor antigens in the past few years, principally in melanoma, led to the surprising finding that many melanoma antigens were derived from normal proteins of melanocyte lineage cells (reviewed in Houghton [9]). This suggests that cancer recognition by the immune system is self-recognition, and that many of the features of a successful cancer immunotherapy will resemble autoimmunity. Hence, the search for immunotherapies for prostatic cancer has included a search for normal self-antigens in the prostate. The goal of prostate cancer immunotherapy has therefore included the goal of inducing autoimmune prostatitis. Thus, our observation that this phenomenon may be occurring in some men with chronic prostatitis is interesting both in the hopes of explaining a baffling chronic condition as well as providing potential targets for prostate cancer immunotherapy.

Several investigators have provided evidence that prostatic antigens can be recognized by T cells. Liu et al. [10] demonstrated in rats that vaccination with a syngeneic prostate homogenate could induce a T-cell immune response to prostate steroid-binding protein (PSBP). This protein, like PSA in humans, is a major secretory product of the rat prostate, but no homologue in humans exists. Vaccination of rats with purified PBSP induced a vigorous antibody and T-cell response and induced inflammatory infiltrates destructive to the prostatic epithelium in some animals. Fong et al. [11] immunized rats with a vaccinia virus expressing human PAP and demonstrated that an inflammatory prostatitis in animals could be engendered. A vaccinia construct containing rat PAP could not induce prostatitis in vaccinated animals. This suggests that a T-cell response to self prostatic antigens can be induced in rats.

In human studies, the culture of T cells demonstrating clear specificity for prostatic antigens has been very difficult. All human tumor antigens recognized by T cells have been identified by the production of such specific T-cell lines, either directly from human tumors or from the peripheral blood of cancer patients, and by the subsequent identification of the antigen being recognized using a variety of techniques. Since prostate specific T-cell lines have not been reported by these methods, investigators have identified known proteins that are specifically expressed by the prostate and have attempted to prove that these proteins or peptides derived therefrom are potential antigens for T lymphocytes. It is possible to induce specific T-cell lines from normal volunteers by in vitro stimulation of lymphocytes with synthetic peptides and interleukin-2 to expand reactive T cells recognizing the peptide [12]. Using such an approach, PSA has been identified as a potential antigen for T cells, principally in normal volunteers of defined Human Leukocyte Antigen (HLA) haplotypes [13–16]. We examined a group of HLA-A2 prostate cancer patients and found similar reactivity with one PSA peptide in only 1 of 7 patients [17].

Other prostatic protein antigens have been explored as potential targets for T cells. Peshwa et al. described CD8 T-cell recognition of peptides derived from PAP [18]. Prostate specific membrane antigen (PSMA), a transmembrane prostate-specific protein, also has been demonstrated to contain antigenic sequences that can be used to generate peptide-specific T cells [19]. These preclinical studies have been performed to support various trials of prostatic cancer immunotherapy with antigen-pulsed dendritic cells [20,21] or vaccination with a recombinant vaccinia virus expressing human PSA [22]. These early clinical trials are designed to induce a prostate-specific immune response presumably directed against metastatic prostate cancer deposits as well as the intact prostate gland. Our data support the notion that PSA may be a target of a human immune response in some men with chronic prostatitis/chronic pelvic pain syndrome, suggesting that attempts to target this antigen in prostate cancer may be successful.

In summary, the data are consistent with the interpretation that some men with chronic prostatitis/chronic pelvic pain syndrome have an autoimmune component to their disease and that PSA may be a normal self-antigen against which this immune response may be directed. In order to establish this definitively will require the culture of T-cell lines specific for PSA, identification of the PSA peptide epitopes being recognized and the HLA haplotypes used to present the peptides, and the determination of precursor frequency of peptide-specific T cells in normals and patients through the course of the disease.

ACKNOWLEDGMENTS

contributions and his contribution to the lives of all of us fortunate enough to know him.

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Antigen Processing and Presentation Gene Expression in Normal, Primary and Metastatic Prostate Tumor Cell Lines

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Running title: TAP and LMP gene expression in prostate lines

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ABSTRACT

In order for immunotherapy to be effective, cytotoxic T lymphocytes (CTL) must recognize target peptides presented on the tumor cells in the context of major histocompatability complex (MHC) Class I molecules. Defective or diminished expression of antigen processing (LMP-2 and LMP-7) and transporters associated with antigen processing (TAP-1 and TAP-2) genes can decrease the cell surface expression of MHC Class I and alter recognition. We utilized reverse transcriptase-polymerase chain reaction (RT-PCR) to analyze LMP-2, LMP-7, TAP-1 and TAP-2 gene expression in normal prostate epithelial and primary prostatic tumor cell lines recently established from the same individual and the long-term cultured metastatic prostate tumor cell lines (DU-145, LNCaP and PC-3). We further investigated the capacity of interferon-γ (IFN-γ) to upregulate the expression of these genes by the cell lines. LMP and TAP gene expression was present in cell lines derived from normal prostate epithelial cells and primary prostate tumor cells and upregulated by IFN-γ treatment. Two of the metastatic prostate tumor cell lines (DU-145 and PC-3) expressed LMP and TAP genes and responded to interferon treatment. The third cell line, LNCaP, had low to undetectable levels of LMP and TAP genes and interferon treatment did not increase the expression of these genes. Our data indicates that prostate tumor cells have intact antigen processing and presenting machinery and should be capable of presenting tumor antigens by HLA Class I molecules to cytotoxic T lymphocytes.

Key Words: Antigen processing, transporter associated with antigen presentation, prostatic cancer, interferon-γ

INTRODUCTION

The ability of antigen presenting cells (APC) to generate antitumor activity by helper and cytotoxic T lymphocytes depends on their capacity to take up and process potential tumor antigens to peptides. These peptides are presented in the context of major histocompatibility complex (MHC) Class I and Class II molecules and interact with cognate T cell receptor on CD4 and CD8 T lymphocytes [1]. T lymphocytes are capable of recognizing and attacking the tumor cell provided the targeted peptide is expressed by the relevant MHC Class I or Class II molecule on the surface of the tumor cell.

Diminished or absent expression of MHC Class I molecules on the surface of tumor cells is one mechanism whereby a tumor cell can evade the immune system[2, 3]. There are a number of reports where this has been documented in various tumor cells or in tumor cell lines, including prostate tumor cells [3-5]. Sanda et al. reported a loss of MHC Class I expression in two of five metastastic prostate tumor cell lines and under-expression of TAP-2 mRNA in one of the cell lines, PPC [6]. Bander et al. utilized immunohistochemistry to analyze MHC Class I cell surface expression with frozen tissue sections of benign prostatic hyperplasia (BPH) and metastatic prostate carcinoma (PCa)[7]. Each of the BPH samples was positive for MHC Class I expression, whereas 50% of the PCa were negative for MHC Class I. Modulation of MHC Class I and Class II cell surface expression on metastatic prostate tumor cell lines by interferon-alpha (IFNα) and IFNγ was also determined. PC-3 and DU-145 had baseline levels of MHC Class I and expression was upregulated by IFN treatment whereas, LNCaP expressed low levels of MHC Class I and was not responsive to IFNα or IFNγ treatment. Blades

et al. reported a similar finding of normal MHC Class I expression on prostate tissue samples obtained from BPH and allele-specific loss (85%) or absent (34%) MHC Class I expression in metastatic prostate carcinoma tissue [8].

As noted above, there is a single report that examined the expression of TAP-2, a transporter associated with antigen processing gene, in long-term cultured, metastatic prostate tumor cell lines [6]. In addition to examining TAP-2 gene expression, we analyzed other MHC genes involved in antigen processing; TAP-1, LMP-2, and LMP-7 in three metastatic prostate cell lines as well as in normal prostate epithelial, seminal vesicle epithelial and primary prostate carcinoma cell lines derived from two patients undergoing radical prostatectomy [9]. The effect of IFN-γ on the expression of antigen processing (LMP-2 and LMP-7) and peptide transport (TAP-1 and TAP-2) genes in these cell lines was also determined.

MATERIALS AND METHODS

Cell Lines

Immortalized cell lines derived from normal prostate (1532NPTX and 1542NPTX), normal seminal vesicle (1542SVTX) and localized tumor (1532CP2TX and 1542CP3TX) were cultured as previously described [9]. These cell lines were created by preparing single cell suspensions from prostate tissue following prostatectomy. The cells were immortalized with a recombinant retrovirus containing the E6 and E7 early genes of human papilloma virus serotype 16 and the selectable marker, neomycin phosphotransferase. Clones of the immortalized cells were prepared by culturing the cells in conditions of less

than one cell/well. Loss of heterozygosity in combination with histochemical and flow cytometric analysis was used to distinguish cell lines derived from tumor cells versus normal prostate epithelium. Immortalized cell lines were cultured and passaged using methods and medium as described previously. Metastatic prostate cell lines, LNCaP, PC-3 and DU145 were grown continuous culture in cRPMI (RPMI-1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% human AB serum (Gemini Bio-Products, Calabasas, CA)) in T-75 tissue culture flasks.

Induction of Antigen Processing Genes

To determine if IFN-γ upregulated expression of MHC antigen processing and transport genes the cell lines were cultured with recombinant human IFN-γ (Genzyme, Cambridge, MA). Briefly, immortalized prostate cell lines and metastatic tumor cells lines were cultured in cRPMI with IFN-γ at concentrations of 0, 100 or 500 U/ml for 72 hours. The cells were harvested with trypsin and counted. Two million cells were pelleted by centrifugation. The supernatant was removed and the cell pellets were snap-frozen and stored at -80°C.

RNA Extraction and cDNA synthesis

Total RNA was extracted from the frozen cell pellets per manufacturers instructions (Qiagen, Santa Clarita, CA). Briefly, frozen cell pellets were rapidly thawed in a lysis buffer containing guanidinium isothiocyanate. Genomic DNA was sheared and viscosity was reduced by applying the sample to a shredding column (Qiagen, Santa Clarita, CA). Ethanol was added to the eluate and the sample was applied to a silica-gel-based membrane. The RNA was washed in the appropriate buffers and eluted in sterile H_2O.

RNA was transcribed into cDNA by mixing 3 μl RNA with a cocktail containing: 2.5 U/μl MuLV (Murine Leukemia Virus) reverse transcriptase, 1 U/μl RNAse inhibitor (originally isolated from human placenta), 1 mM each dATP, dGTP, dCTP, and dTTP (deoxyribonucleoside triphosphates), PCR buffer, 5 mM $MgCl_2$, and 2.5 μM random hexamers (Perkin Elmer, Foster City, CA). RNA was reverse transcribed by incubation at 42°C for 15 minutes, denatured by incubation at 99°C for 5 minutes, and cooled to 5°C.

Polymerase Chain Reaction

cDNA was serially diluted (1:10, 1:50, and 1:100) and aliquotted onto 96 well PCR trays in a volume of 2 ul/well. A master mix of 0.2 mM dNTP, PCR buffer, 1.5 mM Mg_2Cl, sterile H_2O, 50 pmol of each primer (Operon Technologies, Alameda, CA) and 1 U Taq Gold polymerase (Perkin Elmer, Foster City, CA) were added to each well. Thirty cyles of PCR was performed with the following conditions: 94°C denaturing, 30 seconds; 58°C annealing, 30 seconds; 72°C extension, one minute; at the end of thirty cycles, an additional ten minute extension. The primer sequences for each gene analyzed were as follows: Tubulin (5'TCCTTCAACACCTTCTTCAG, 5'TGGCCTCATTGTCTACCAT G); TAP-1 (5'CCGCCTCACTGACTGGATTC, 5'GCACGTGGCCCATGGTGT TGTTATAG); TAP-2 (5'GCCGAGCATGAAGTCTG, 5'CCACGCTCTCCTGG TAGATC); LMP-2 (5'GGCAGTGGAGTTTGACGG, 5'GGCTGTCGAGTCAG CATTC); LMP-7 (5'TCTACTACGTGGATGAACATGG, 5'TTGATTGGCTTC CCGGTACTG). PCR products were analyzed by 6% polyacrylamide gel electrophoresis stained with ethidium bromide and visualized under an ultraviolet light.

Results

Antigen Processing and Transporter Gene Expression in Normal Prostate Epithelial and Primary Prostate Tumor Cell Lines

Normal prostate epithelial cell lines were assessed for LMP and TAP gene expression by RT-PCR. As shown in figure 1a and 1c, normal prostate cell lines derived from both patient samples express LMP and TAP genes. Primary prostate tumor cell lines were derived from the same patients and were similarly analyzed for antigen processing and presentation gene expression. LMP-2, LMP-7, TAP-1 and TAP-2 gene expression was detected in both primary prostate tumor cell lines as shown in figure 1b and 1d. Expression of the housekeeping gene, tubulin, was used as a semi-quantitative control.

Effects of IFN-γ Treatment on Antigen Processing and Transporter Gene Expression in Primary Prostate Tumor and Normal Prostate Epithelial Cell Lines

Expression of LMP and TAP genes can be induced by treatment with IFN-γ. We determined whether IFN-γ would increase the expression of antigen processing and transport genes in normal prostate epithelial and primary prostate tumor cell lines by culturing the cells for seventy-two hours with 100 or 500 U/ml of IFN-γ. Cell lines derived from normal and primary prostate cancer cells were responsive to treatment with IFN-γ as shown in figure 1.

Antigen Processing and Transporter Gene Expression in Metastatic Prostate Tumor Cell Lines

In addition to detecting LMP and TAP gene expression in normal and primary prostate tumor cell lines which have been in continuous culture for more than one year, we analyzed LMP and TAP gene expression in three metastatic prostate tumor cell lines which were generated more than twenty years ago. DU-

145, LNCaP and PC-3 cells all expressed TAP-1 and TAP-2 (figure 2), although less PCR product was detected in the metastatic prostate cell lines compared to the normal and primary tumor lines. Detectable levels of LMP-2 and LMP-7 were seen in DU-145 and PC-3 cells, again at a lower intensity than seen for normal and primary prostate cell lines. LMP-2 and LMP-7 gene expression was not detectable in LNCaP cells.

Effects of IFN-γ Treatment on Antigen Processing and Transporter Gene Expression in Metastatic Prostate Tumor Cell Lines

The effect of IFN-γ on LMP and TAP gene expression was also assessed in three metastatic prostate cell lines, DU-145, PC-3 and LNCaP (Figure 2). IFN-γ treatment increased antigen processing and transport gene expression in both DU-145 and PC-3 cells. Consistent with other reports, LNCaP cells were not responsive to IFN-γ.

Discussion

Prostate cancer is the second most common type of cancer found in American men. The American Cancer Society estimates that there will be 179,300 new cases of prostate cancer diagnosed in the United States in 1999, and about 37,000 men will die of this disease. Primary prostate carcinoma will account for approximately 60% or 107,500 of these newly diagnosed cases. Immunotherapy approaches for the treatment of prostate cancer include generating APC *ex vivo*, pulsing them with known candidate prostate tumor antigens (such as peptides derived from PSMA) and infusing them back into the patient [10, 11]. The effectiveness of these treatment modalities is dependent upon

the ability of cytotoxic T lymphocytes to recognize tumor peptide antigens presented in the context of MHC Class I molecules on the tumor cells.

Endogenously synthesized proteins that are potential tumor antigens are processed into peptides by the multicatalytic proteasome complex containing the interferon inducible subunits, LMP-2 and LMP-7 [1]. Once generated, the peptides are shuttled across the endoplasmic reticulum by a heterodimeric complex composed of the transporters associated with antigen processing (TAP-1 and TAP-2). Within the ER, the TAP complex is physically associated with nascent MHC Class I molecules and mediates the loading of peptides into the binding cleft of MHC Class I. Downregulated expression of either the proteasome, LMP-2 and LMP-7, or transporter associated with antigen processing, TAP-1 and TAP-2, can result in defective or diminished MHC Class I expression on the surface of tumor cells.

We analyzed proteasome (LMP-2 and LMP-7) and transporter associated with antigen processing (TAP-1 and TAP-2) gene expression in normal prostate epithelial, seminal vesicle epithelial and primary prostate carcinoma cell lines derived from two patients undergoing radical prostatectomy [9] by reverse transcriptase-polymerase chain reaction (RT-PCR). We report here that antigen processing gene expression is intact in the cell lines derived from primary prostate tumor carcinoma and normal prostate epithelial cell lines. In addition, these cell lines were responsive to treatment with IFN-γ. In order to determine whether immortalization affected the expression of antigen processing and presentation genes, short-term unimmortalized cultures of the normal and primary prostate

carcinoma cells were analyzed. Levels of TAP and LMP gene expression were intact in these short-term cultured cells (data not shown).

In addition to the normal and primary prostate tumor cells, we analyzed proteasome (LMP-2 and LMP-7) and transporter associated with antigen processing (TAP-1 and TAP-2) gene expression in three metastatic prostate tumor cell lines (DU-145, LNCaP and PC-3). Our findings extend those of Sanda et al. who reported that TAP-2 gene expression was intact in DU-145 and PC-3 and defective in LNCaP. We show that DU-145 and PC-3 expressed low, but detectable levels of antigen processing genes and were responsive to IFN-γ, whereas LNCaP expressed very low levels of antigen processing gene RNA which was modestly increased by treatment with IFN-γ.

Blades et al. assessed surface expression of MHC Class I molecules in prostate samples by immunohistochemistry. A complete loss of MHC Class I expression was seen in 34% of prostate carcinoma samples. No correlation between loss of MHC Class I expression and stage of disease was seen in the study by Blades [8]. In contrast, Klein et al. reported intact surface expression of β-2M on 88% of well-differentiated prostate adenocarcinoma whereas only 16% of poorly differentiated tumors were positive [4]. Bright et al. used flow cytoemetric analysis to demonstrate the expression of MHC Class I on the surface of immortalized normal and primary prostate cell lines [9].

Immunotherapy relies upon CTL recognition of tumor peptide antigens presented by MHC Class I on the surface of tumor cells. Our data show that prostate tumor cell lines should be capable of presenting tumor antigens on MHC Class I and should be targeted by CTL, since primary prostate cancer cell lines

and two of three metastatic prostate cancer cell lines analyzed expressed antigen processing machinery and were responsive to treatment with IFN-γ. These data lend support for continued immunotherapeutic approaches using dendritic cells armed with putative prostate tumor antigens, particularly for the treatment of minimal residual disease or prevention of recurrent disease following prostatectomy.

Acknowledgments

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Figure Legends

Figure 1. **TAP and LMP gene expression in normal and primary prostate cancer cell lines.** Normal and primary prostate cancer cell lines were established from two patients undergoing radical prostatectomy for primary prostate adenocarcinoma as described in the methods. The cells were plated in six well plates and cultured for 48 hrs. with 0, 100, or 500 U/ml IFN-γ. The cells were harvested and counted. Two million cells were removed and a cell pellet was prepared by centrifugation. The supernatant was removed and the cell pellet was snap frozen and stored <-80°C. Total RNA was isolated from the cell pellets and cDNA synthesized. The cDNA was serially diluted 1:1, 1:10, 1:50, and 1:100 and PCR performed using the primers as indicated. PCR products were analyzed by 6% PAGE, stained with ethidium bromide, visualized under UV light and photographed. Both patient cell lines expressed LMP and TAP genes shown in lanes 1-4. The cell lines were responsive to treatment with IFN-γ as shown in lanes 5-12. Tubulin, a constitutively expressed gene, was used as a control.

Figure 2. **TAP and LMP gene expression in metastatic prostate cancer cell lines.** Longterm metastatic prostate cancer cell lines were cultured with 0, 100 or 500 U/ml IFN-γ and harvested as described in figure 1. DU-145 and PC-3 (figure 2a and 2c) cells express low but detectable levels of all genes analyzed (lanes 1-4) and were responsive to treatment with IFN-γ (lanes 5-12). LNCaP (figure 2b) cells did not express detectable levels of LMP-2 and LMP-7 and was not responsive to treatment with IFN-γ (lanes 5-12).

Figure 1

a.   1532NPTX

IFN-γ   0   100   500

Tubulin

TAP-1

TAP-2

LMP-2

LMP-7

b.   1532CP2TX

IFN-γ   0   100   500

Tubulin

TAP-1

TAP-2

LMP-2

LMP-7

c.   1542NPTX

IFN-γ   0   100   500

Tubulin

TAP-1

TAP-2

LMP-2

LMP-7

d.   1542CP3TX

IFN-γ   0   100   500

Tubulin

TAP-1

TAP-2

LMP-2

LMP-7

Figure 2

a.
DU-145

b.
LNCaP

c.
PC-3

| IFN-γ | 0 | 100 | 500 | | 0 | 100 | 500 | | 0 | 100 | 500 |

Tubulin

TAP-1

TAP-2

LMP-2

LMP-7
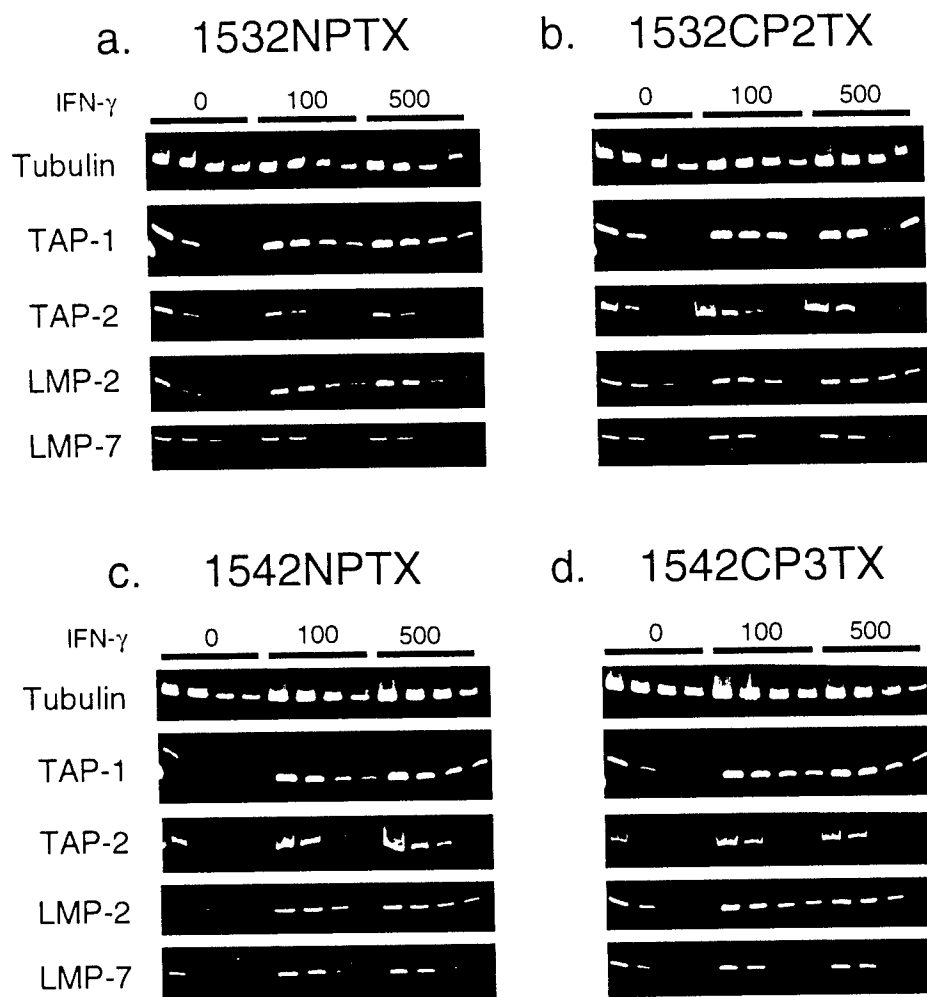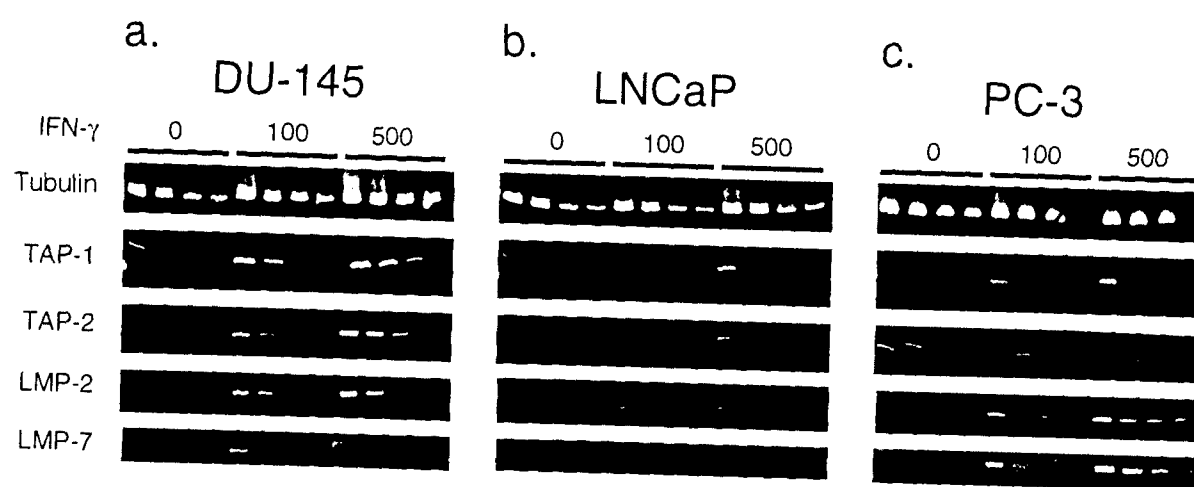
In Preparation

CD4 and CD8 T lymphocyte recognition of prostate specific antigen in a patient with granulomatous prostatitis

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Introduction

Prostate specific antigen (PSA) is a 40K protein from the family of kallikreins that is exclusively expressed in prostate tissue {Papsidero, Kuriyama, et al. 1981 907 /id}. Several lines of evidence support the possibility that PSA may be a potential target for immunotherapy of prostate cancer. PSA is a normal prostate-specific protein and, in other human malignancies, T cell antigens have been well-defined and are often derived from such normal self proteins expressed by mature cells of the same lineage as the tumor {Rosenberg 1997 936 /id} {Boon, Coulie, et al. 1997 937 /id}. Second, PSA is expressed at high levels in the majority of prostate tumor cells {Oesterling 1993 172 /id}. Third, a T cell response to PSA or derivative peptides have been demonstrated in normal donors and prostate cancer patients using whole protein, polynucleotide-based vaccines, or PSA-oligoepitope peptide vaccines {Meidenbauer, Harris, et al. 2000 906 /id} {Kim, Trivedi, et al. 1998 848 /id} {Heiser, Dahm, et al. 2000 855 /id} {Correale, Walmsley, et al. 1998 767 /id} {Alexander, Brady, et al. 1998 718 /id}. Finally, the potential danger of developing an autoimmune response to normal tissues is limited because the prostate gland is not essential for life beyond the reproductive years and cancer of the prostate is typically a disease of older men.

One approach to the study of prostate cancer immunotherapy has been to identify methods to induce inflammatory infiltrates in the normal prostate of animals (prostatitis) and to search for prostatic antigens recognized by T lymphocytes in such animals. Liu et al {Liu, Chatta, et al. 1997 713 /id} identified prostate steroid binding protein, a major secretory protein of the rat prostate, as a T cell antigen using such an approach. There is, however, no homologue for PSBP in humans. This work demonstrates that it is possible

for a normal secretory product of the murine prostate to be a T cell antigen and supports the hypothesis that a normal human prostate secretory product, PSA, is a logical choice as a potential T cell antigen.

Given the similarity between cancer immunotherapy and autoimmunity we have attempted to determine if autoimmune recognition of prostate tissue could be occurring in other human conditions in order to search for prostate antigens recognized by T lymphocytes. We have principally studied patients with chronic prostatitis or chronic pelvic pain syndrome, a common but poorly characterized condition without a clearly defined etiology {Alexander & Trissel 1996 449 /id}. We reasoned that the study of a natural disease process characterized by inflammation of the prostate would be a better chance for identifying prostate antigens than by studying normal donors. We have found evidence that some men with chronic prostatitis may have an autoimmune component to their disease since they manifest a peripheral CD4 T cell proliferative response to prostate secretions {Alexander, Brady, et al. 1997 712 /id}, pro-inflammatory cytokines are found in the prostatic secretions in these patients {Alexander, Ponniah, et al. 1998 750 /id} {Nadler, Koch, et al. 2000 939 /id} and a proliferative T cell response to PSA is found in some men with this condition {Ponniah, Arah, et al. 2000 870 /id}. These data suggest that chronic prostatitis may have an autoimmune component in some patients, and that PSA is a candidate self antigen that may be involved in the autoimmune process.

In this study, we screened cultured PBMC from several patients with chronic prostatitis for IFNγ secretion after exposure to exogenous purified human PSA. One of these men, patient Pr115, was found to have granulomatous prostatitis on biopsy of the gland and had high levels of IFNγ in response to PSA in his PBMC cultures. Long-term

CD4 and CD8 T cell lines and clones were developed from this patient. HLA restriction of these T cell lines was determined using a panel of blocking antibodies against different HLA molecules, and confirmed using HLA matched and mismatched stimulator cells. The ability of these T cell lines to recognize naturally processed PSA was demonstrated using a panel of EBV-B cell lines with known HLA types that were engineered to express endogenous PSA. The data show that PSA can be an antigen both for CD4 and CD8 T lymphocytes.

Methods

Reagents: Purified human PSA, β-microseminoprotein and prostatic acid phosphatase

were purchased from Fitzgerald Industries International Inc. (Concord, MA),

recombinant human IL2 was from Chiron Corporation (Emeryville, CA), rhuIL-4, GM-

CSF, TNF-α, IL-7 were purchased from R&D Systems (Minneapolis, MN). Human AB

male serum was obtained from Gemini Bio-Products (Woodland CA), human AB female

serum was purchased from Omega Scientific, Inc. (Tarzana, CA). Lymphocyte separation

medium (LSA) was obtained from ICN Pharmaceutical, Inc. (Costa Mesa, CA).

Monoclonal antibodies W6/32 (anti-HLA A,B,C) and L243 (anti-HLA-DR) (American

Type Culture Collection, Manassas, VA) for blocking studies were produced by culturing

hybridoma cells, and antibodies purified from conditioned culture supernatant using

protein A/G columns (Pierce, Rockford, IL). Purified mouse IgG2a used as isotype

control was from R&D Systems. Monoclonal antibodies against HLA-A3, -A24, -B7, -

DR4, -DR15, DR52 were obtained from One Lambda Inc. (Canoga Park, CA), mouse

IgG1 and IgM were from Sigma (St. Louis, MO).

Cell lines: EBV-B transformed homozygous B cell lines IHW 9008 and IHW 9033 were

obtained from International Histocompatibility Workshop bank. EBV-B cell line Pr97

was developed in our laboratory from PBMC by incubation with virus-containing

supernatant derived from B-98-5 cell line. All B cell lines were maintained in complete

Iscove's Modified Dulbecco's media (supplemented with 2mM L-glutamine, 0.1 mM

MEM non-essential amino acids, 1 mM MEM sodium pyruvate, 100 U/ml penicillin, 100

μg/ml streptomycin, 5.5×10^{-5} M 2-Mercaptoethanol (all reagents from Life Technologies,

Gaithersburg, MD)) in the presence of 5% human female AB serum. The human prostate

carcinoma cell line LNCaP was purchased from American Type Culture Collection and maintained in complete RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% human male AB serum.

Patient: Peripheral blood mononuclear cells (PBMC) from patients with chronic prostatitis/chronic pain syndrome were prepared by leukopheresis under an Institutional Review Board-approved protocol at the University of Maryland. PBMC were harvested using LSM gradient centrifugation, and cryopreserved in liquid N_2. HLA typing was performed in the American Red Cross Natioanal Histocompatibility Laboratory at the University of Maryland Medical System. The following HLA phenotype was found for patient Pr115: HLA-A*0301/2402; -B*0702/1501; -Cw*0303/0702; -DRβ1*1501/04; - DRβ4*01; - DRβ5*0101.

Primary stimulation with PSA: PBMC from patients with chronic prostatitis were stimulated with antigen as described earlier ({Coligan, Kruisbeek, et al. 1997 783 /id} with modifications. Briefly, 2 ml of $2x10^6$ cells/ml unfractioned PBMC ($4x10^6$ cells) were placed into wells of 24-well flat-bottom microtiter plate in the presence of 10 μg/ml of PSA. Cells were incubated for 7 days in a humidified 37°C, 5%CO_2 in complete Iscove's Modified Dulbecco's media and 5% human female AB serum. Human rIL-2 was added at 30 U/ml on day 2 and day 5. After 7 days of culture, cells were harvested and centrifuged through LSM density gradient. Cells recovered at interface were washed twice, and plated into 24-well microtiter plate at $1x10^6$ cells/ml in culture medium. Unfractioned autologous PBMC were γ-irradiated at 3,000 rad, and added at $4x10^6$ cells/well to T cell cultures. Cells were incubated for additional 7 days in a humidified 37°C, 5% CO_2 incubator. At the end of culture, cells were centrifuged through a LSM

gradient, and were used for experiments or continued to culture and cloned by limiting dilution.

Generation of PSA-specific T cells lines After 3 round of stimulation with antigen, T cells lines were cloned by limiting dilution. T cells at 30, 10, 3.3 and 1.1 cells/well were added into 96-well round-bottom microtiter plates containing $1x10^5$ irradiated autologous PBMC, PSA at 10 µg/ml, rIL-2 at 30 U/ml and rIL-7 at 10 ng/ml. Cultures were incubated for 10 days, 50 µl of media was replaced on day 4 and day 7 with fresh IL-2 and IL-7. On day 10, 50 µl media was replaced with fresh irradiated autologous PBMC $(1x10^5)$, PSA at 10 µg/ml, rIL-2 at 30 U/ml. Growing colonies were transferred into 24 – well plates, and maintained as described below.

Maintentance of T cell lines: T cell lines were plated at $5x10^5$ cells/well in 24-well microtiter plates together with irradiated autologous PBMC at $2x10^6$ cells/ml, PSA at 10 µg/ml, and rIL-7 at 10 ng/ml; rIL-2 at 30 U/ml was added on day 2. Cells were cultured for 14-21 days, media containing rIL-2 at 30 U/ml and rIL-7 at 10 ng/ml was replaced every 3-5 days. After several cycles of stimulation with antigen in the presence of autologous PBMC, CD4 T cell line 2B1 stopped to grow; addition of autologous EBV-B cells did not rescue T cells from dying. To stimulate cell expansion, we have developed an alternative protocol utilizing the combination of autologous dendritic cells (DC) pulsed with antigen and allogeneic feeder PBMC. DC were prepared from PBMC by negative selection using anti-CD2 and anti-CD19 immunomagnetic beads (Dynal). CD14-enriched cells were cultured in the presence of IL-4 and GM-CSF(1000 U/ml) for 6 days, and matured by adding TNF-α (30 ng/ml) during 3 last days of incubation ({Berlyn, Ponniah, et al. 1999 805 /id}). DCs were pulsed with PSA at 5 µg/ml for 1-3

days, then washed, irradiated at 1,000 rad and mixed (1×10^5 cells/well) with T cells

(5×10^5 cells/well) in 24-well tissue culture plates. Irradiated allogeneic PBMC pooled

from at least 3 different donors {Kahle, Wernet, et al. 1981 919 /id} were added at 2×10^6

cells/well, and rIL-7 was added at 10 ng/ml. Cells were cultured for 14-21 days, media

containing rIL-2 at 30 U/ml and rIL-7 at 10 ng/ml was replaced every 3-5 days. Neither

antigen-pulsed DC nor allogenenic PBMC alone were able to stimulate a long-term

expansion of CD4 T cell line 2B1.

Flow cytometry analysis: The phenotype of surface markers expressed by T cell line was

determined by flow cytometry as previously described {Alexander, Fitzgerald, et al. 1995

285 /id}. The mAbs used were anti-CD4-FITC/anti-CD8-PE, anti-TcR α/β-FITC and

IgG2a-FITC/IgG2a-PE (Caltag Laboratories, Burlingeim, CA).

Retroviral transduction The recombinant PSA/pLNSX and control pLNSX retroviral

constructs alone with packaging line PA317 were kindly provided by Dr. Jeffrey Schlom

(Laboratory of Tumor Immunology and Biology, NIH, Bethesda, MD). EBV-B cell lines

were co-cultured with retroviral supernatants followed by selection in the presence of

G418 (1 mg/ml). Antibiotic-resistant EBV-B cell lines were expanded in culture. PSA

expression was confirmed by measuring PSA in culture supernatants by ELISA using

monoclonal anti-PSA coating antibody and polyclonal anti-PSA detecting antibody

(Fitzerald Indusries International Inc.) followed by donkey anti-rabbit IgG F(ab')2:

alkiline phosphatase conjugate (Research Diagnostics Inc., Flanders, NJ).

Recombinant vaccinia virus. Recombinant vaccinia virus containing the full length gene

for PSA and wild type virus were kind gift of Dr. Jeffrey Schlom {Hodge, Schlom, et al.

1995 443 /id}. EBV-B cells were plated at 1×10^6 cells/ml in culture media in 24-well

plates. Wild type or recombinant PSA-vaccinia virus (5×10^6 plaque-forming units per 1×10^6 EBV-B cells) were added to the culture and incubated for 2 h at 37°C. Cells were washed twice and mixed with T cells as described below. PSA expression by infected EBV-B cell lines was determined in supernatants at 48 h by ELISA.

Measurement of PSA-specific T cell response by cytokine secretion. After 14-21 days of stimulation with antigen, T cells were harvested, washed 4 times with culture media, and plated into round-bottom 96-well microtiter plates at 5×10^4 cells/well. Autologous PBMC were irradiated at 3,300 rad and added at 1×10^5 cells/well, EBV-B cells were irradiated at 6,000 rad and added at 1×10^4 cells/well; PSA was added at 10 μg/ml. In some experiments, adherent PBMC pulsed overnight with antigen were used for stimulation. Antibodies against HLA-A,B,C, HLA-DR or control IgG2a were added at 5-10 μg/ml, antibodies against HLA-A3, -A24, -B7, -DR15, -DR4 or control IgG1 and IgM were added at 1/40 dilution. After 48h, culture supernatants were harvested, and cytokine secretion in the supernatants was measured by ELISA using pairs of capture and biotinylated detecting mAbs (all reagents from Endogen).

Results

IFNγ secretion in vitro by cultured T cells from prostatitis patients

It has been previously showed in our laboratory that some patients with chronic

prostatitis/chronic pelvic pain syndrome had a recall T cell proliferative response specific

for PSA that was absent in normal male donors {Ponniah, Arah, et al. 2000 870 /id}. To

characterize this phenomenon further, we wished to determine whether peripheral blood

mononuclear cells (PBMC) from such patients would secrete IFNγ in response to PSA.

PBMC were stimulated for 14 days with purified PSA obtained from seminal plasma,

then T cells were harvested and re-stimulated with PSA in the presence of irradiated

autologous PBMC. IFNγ secretion in the culture supernatants was measured at 48h by

ELISA. In control wells, T cells were stimulated by plastic-bound anti-CD3 mAbs. In

Figure 1, a representative experiment shows that T cells from one patient (Pr115)

secreted a significant level of IFNγ in response to PSA, whereas the response of another

patient with chronic prostatitis (Pr90) was low (Fig.1A). Cells from both patients

demonstrated equally strong response to anti-CD3 mAbs (Fig.1B). PSA-specific

responses in Pr115 were partially blocked by monoclonal antibodies directed against

monomorphic class I and class II (DR) antibodies, suggesting that both CD4+ and CD8+

T cells are responding to PSA (data not shown).

Characterization of CD4+ PSA-specific T cell line 2B1 from patient Pr115.

T cell lines were generated from PBMC of patient Pr115 by repeated stimulation

with purified PSA. Several T cell clones derived from these cultures by limiting dilution

retained their PSA reactivity as measured by increase in IFNγ secretion. T cell line 2B1

was more than 90% CD4+ and TcR α/β+ as determined by flow cytometry (data not

shown). 2B1 T cells secreted IFNγ in response to PSA presented by irradiated autologous

PBMC, but not by allogeneic HLA-mismatched PBMC (Fig. 2A). The increase in IFNγ

was also seen when 2B1 T cells were stimulated by autologous PBMC pulsed with

lysates prepared from PSA-secreting prostate tumor cell line LNCaP (Fig.2A).

The two other major secretory products of the prostate found in semen are prostatic acid

phosphatase and β-microseminoprotein {Lilja & Abrahamsson 1988 388 /id}. No

secretion of IFNγ by 2B1 was observed if purified prostatic acid phosphatase or β-

microseminoprotein were substituted for PSA (Fig. 2B). This demonstrates that the

recognition by 2B1 of PSA is antigen-specific and MHC-restricted.

Antibody blocking studies

PSA-specific IFNγ secretion by 2B1 was blocked by anti HLA-DR monomorphic

monoclonal antibody, but not by anti HLA-A,B,C antibody (Fig.3A). To determine HLA

restriction of 2B1, the stimulation with antigen was performed in the presence of

monoclonal antibodies specific to different HLA-DR serotypes expressed by the patient.

As shown in Figure 3B, blocking was achieved with anti-HLA-DR15 antibody but not

with an antibody to HLA-DR4, DR52 or control IgG1. HLA restriction was also

confirmed using stimulator PBMC from HLA-DRβ1*1501 positive allogeneic donor

(data not shown). These studies demonstrate that 2B1 recognition of PSA is restricted by

HLA-DRβ1*1501.

PSA-vaccinia experiments

To confirm the specificity of T cell line 2B1 we wished to determine if the cells

could recognize PSA made and secreted by APC. We infected EBV-B cell lines of

known HLA type with recombinant vaccinia virus that contained the PSA gene or with

control wild-type virus, and used these cells to stimulate T cells. As shown in Figure 4, T cell line 2B1 secreted IFNγ specifically in response to PSA-expressing HLA-DRβ1*1501 positive EBV-B cell line IHW 9008, but not to PSA-expressing HLA-DRβ1*1501 negative line IHW 9033 or either EBV-B cell line infected with wild type virus. These studies confirm that the response of 2B1 is specific for PSA and restricted by HLA-DRβ1*1501.

Characterization of CD8+ PSA-specific T cell line from patient Pr115.

Among several T cell lines generated from PBMC of patient Pr115 by in vitro restimulation with purified PSA, clone 5H10 was more than 95% CD8+, TcR α/β+ as determined by flow cytometry (data not shown). When stimulated with anti-CD3/anti-CD28 coated immunomagnetic beads, T cell line 5H10 secreted IFNγ, GM-CSF and TNF-α but not IL-4 or IL-10 (Table I). 5H10 secreted IFNγ when exposed to PSA-pulsed autologous PBMC but not unpulsed PBMC as shown in Fig. 5. IFNγ secretion in response to PSA presented by autologous PBMC was blocked by a HLA Class I monomorphic monoclonal antibody, but not by HLA class II antibody (Fig.5A). To determine HLA restriction of T cell line 5H10, a panel of monoclonal antibodies specific to HLA Class I molecules expressed by the patient was used to block PSA-specific IFNγ secretion. As shown on Figure 5B, blocking was achieved with anti-HLA-B7 antibody but not with an antibody to HLA-A3, -A24 or control IgM.

Recognition of endogenous PSA

We wished to determine whether 5H10 could recognize PSA that is expressed, processed and presented as an endogenous protein. We used an EBV-B transformed B cell line from the donor Pr97 that matched patient Pr115 only in HLA-B*0702. These

cells were transduced with retroviral pLNSX vector containing the PSA gene. This cell line expressed PSA endogenously, and secreted it into the medium as determined by PSA ELISA in culture supernatants (data not shown). In Figure 6, CD8+ T cell line 5H10 secreted IFNγ in response to this EBV-B cell line tranduced with PSA-containing vector, but not with control vector, and the response was blocked by a monomorphic anti-HLA Class I antibody and anti-HLA-B7 monoclonal antibody. This demonstrates that the response of 5H10 is specific for PSA and restricted y HLA-B*0702 and that PSA can be processed and presented endogenously to the 5H10 T cell line.

Discussion.

We have previously demonstrated that autoimmunity to PSA or other prostatic proteins may contribute to symptoms in some patients with chronic prostatitis/chronic pelvic pain syndrome {Ponniah, Arah, et al. 2000 870 /id}. In order to prove directly that PSA is a T cell autoantigen, we determined whether the response to PSA in these patients was characterized by increase in secretion of IFNγ. Among six patients with chronic prostatitis that developed recall proliferative responses to PSA in the initial study, three patiens showed a significant and specific increase in IFNγ secretion in response to PSA in secondary cultures. Long-term CD4 and CD8 T cell lines were successfully generated from peripheral blood of one patient that showed exceptionally high level of IFNγ secretion.

CD4 T cell line 2B1 was selected by a significant PSA-specific increase in IFNγ secretion, and showed the restriction to HLA-DRβ1*1501. The PSA specificity of the responses was confirmed using different proteins derived from seminal plasma, as well as recombinant PSA and lysate from PSA-secreting tumor cell line LNCaP. HLA restriction was confirmed using anti-HLA-DR15 monoclonal antibodies, as well as allogeneic stimulator cells from HLA-DRβ1*1501 positive and negative donors. To our knowledge, this is the first demonstration that HLA class II allele is involved in PSA response. CD4 T cell lines specific to predicted HLA-DR4-binding peptide have been described earlier, however the responses of these lines to whole PSA has not been shown {Corman, Sercarz, et al. 1998 849 /id}.

[Despite of the fact that exogenous antigen was used in initial cultures, we were able to establish a long-term PSA-specific CD8 T cell line 5H10.] Short-term CD8 T cell

lines specific to PSA-derived peptides has been described earlier by several groups. CD8+ cytotoxic T cell response to PSA peptides that can bind HLA-A1, A2-, -A3, -A24 and –B53 alleles have been demonstrated in normal donors and prostate cancer patients *in vitro* and *in vivo* {Correale, Walmsley, et al. 1997 667 /id}{Corman, Sercarz, et al. 1998 849 /id}. 'CD8 T cell line 5H10 described in this report showed the restriction to HLA-B*0702. To our knowledge, the HLA-B7-restricted response to PSA has not been reported before.

For CD8 T cell lines, it is important to demonstrate that they can react with target cells that express PSA endogenously. The reactivity of HLA-A2-restricted T cell lines with PSA-expressing HLA-A2 positive prostate tumor cell line LNCaP has been descrived earlier {Correale, Walmsley, et al. 1998 767 /id} whereas PSA-expressing tumor cell lines with HLA types other that HLA-A2 were not available. To overcome this problem, we have generated a panel of EBV-B cell lines from donors with different HLA types that were engineered to express recombinant PSA using retroviral vector containing PSA gene, and confirmed that CD8 T cell line 5H10 described in this study was able to recognized recombinant endogenously expressed PSA. [Ability to recognize prostate tumor cell line LNCaP]. Further characterization of the epitope specificity of generated T cell lines is currently undergoing in our laboratory.

PSA-specific CD4 and CD8 T cell lines described in this study were developed from a patient with granulomatous prostatitis that is characterized by an acute and extensive infiltration of lymphocytes into the prostate. A study is initiated to determine possible association of this relatively rare form of chronic prostatits with HLA-DRβ1*1501 and HLA-B*0702 alleles. Both alleles are often co-expressed in the

ancestral haplotype denoted 7.1 (HLA-A3, B7, TNFα 11b4, DRB1*1501). Susceptibility to multiple sclerosis is clearly associated with HLA-DRB1*1501(reviewed in{Allcock, de la Concha, et al. 1999 918 /id}) and some studies showed its association with HLA-B7 {Madigand, Oger, et al. 1982 920 /id}. We are also undertaking a further characterization of T cell receptor usage in these T cell lines in order to compare it with clonal composition and T cell receptor repertoire of T cells infiltrating prostate gland characterize prostate- infiltrating lymphocytesin HLA-matching patients with granulomatous prostatitis.

Taken together, we have developed CD4 and CD8 PSA-specific T cell lines from the patient with granulomatous prostatitis that were restricted to HLA-DRβ1*1501 or HLA-B*0702 respectively. Our data confirm the autoimmune nature of chronic prostatitis in some patients. It has been demonstrated in several studies in human malignancies that normal self antigens are recognized by T cells in an MHC-restricted fashion suggesting that a successful imunotherapy for cancer will resemble autoimmunity{Houghton 1994 483 /id}. It has been shown in patients with metastatic melanoma that spontaneous regression of tumor, as well as clinical responses after IL-2 therapy correlated with the destruction of normal melanocytes (vitiligo) {Houghton 1994 483 /id} {Rosenberg & White 1996 423 /id}. This implies that a successful immunotherapy for prostate cancer may resemble an autoimmune response to prostate differentiation antigens. Our results suggest that PSA and PSA-derived peptides can be used to develop the effective vaccine for prostate cancer immunotherapy in patients with appropriate HLA type, and extend the number of HLA Class I and Class II alleles that can accommodate the response to PSA.

Tables

Table I. IFN-γ secretion in response to PSA in secondary cultures by PBMC from

prostatitis patients.

Patients	IFN-γ, pg/ml[a]		SI[b]
	PSA	No Ag	
Experiment 1			
Pr94	1937±30	617±34	3.1
Pr115	1252±123	86±120	14.5
Experiment 2			
Pr90	Below detection	Below detection	N/A
Pr115	260±29	35±11	7.4
Experiment 3			
Pr71	238±21	73±26	3.3
Pr73	Below detection	Below detection	N/A
Pr94	196±11	66±10	2.4
Pr115	415±61	191±21	2.2

[a] PBMC from patients with chronic prostatitis were stimulated as described in the legend

to Figure 1. IFN-γ concentration was determined by ELISA on day 2, data are presented

as Mean±SD of duplicates.

[b] The stimulatory index (SI) was calculated by equation: (Response$_{PSA}$/ Response$_{no\ Ag}$)

Table II. Characteristics of T cell clones derived from patient Pr115.

| Clone | IFN-γ secretion in response to PSA[a] pg/ml [% inhibition] | | | | Phenotype[b] (% positive by FACS) | |
| | PSA | | | | | |
	media	Class I mAb	Class II mAb	No Ag	CD4	CD8
2B1	560±51	495±127 [12%]	B. D. [100%]	B. D.	92%	2.5%
5A11	809±81	1206±14 [0%]	561±5 [30%]	443±28	84%	14%
5G12	399±39	329±17 [17%]	B. D. [100%]	137±27	96%	0.5%
5A10	1539±59	B. D. [100%]	1325±209 [14%]	473±84	1%	96%
5H10	1663±156	35±8 [98%]	2612±183 [0%]	322±21	1.6%	94%
5H11	285±2	141±5 [50%]	261±38 [8%]	B. D.	1%	97%

[a] T cell clones were plated at $5x10^4$ cells/well in 96-wel round bottom plate and were stimulated by irradiated autologous PBMC ($1x10^5$ cells/well) in the presence or absence of PSA and monoclonal antibodies to HLA molecules. IFN-γ concentration was determined after 2 days of stimulation by ELISA. Data are presented as Mean±SD of duplicates. Percent of inhibition was calculated by equation:

[1-(PSA response$_{media}$/ PSA response$_{mAb}$)]x100%.

[b] T cell clones were stained by anti-CD4-FITC and CD8-PE mAbs as described in "Materials and Methods".

Figure 1

A. Autologous PBMC
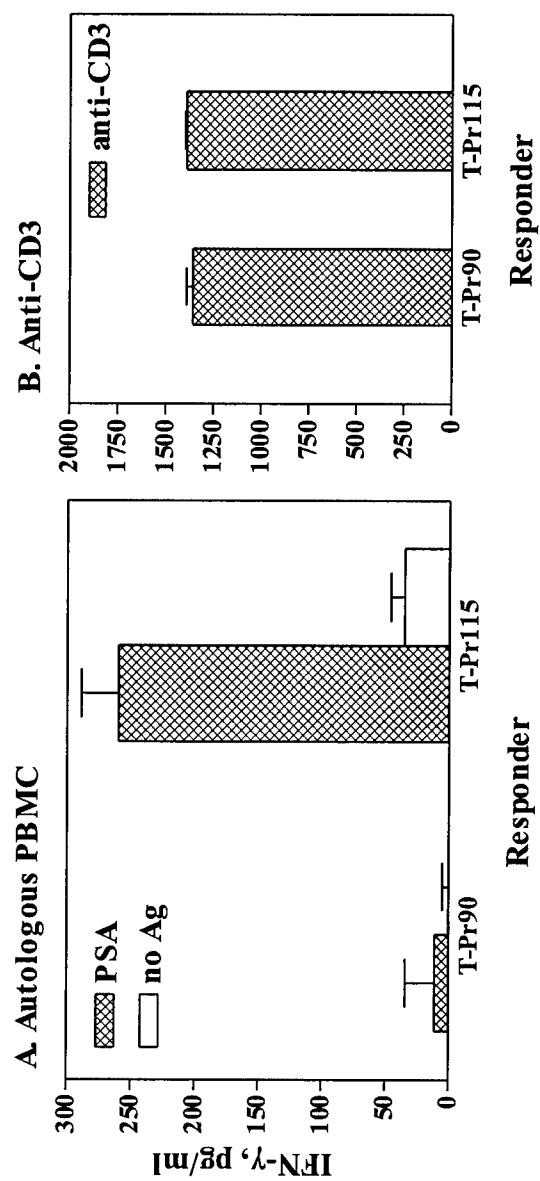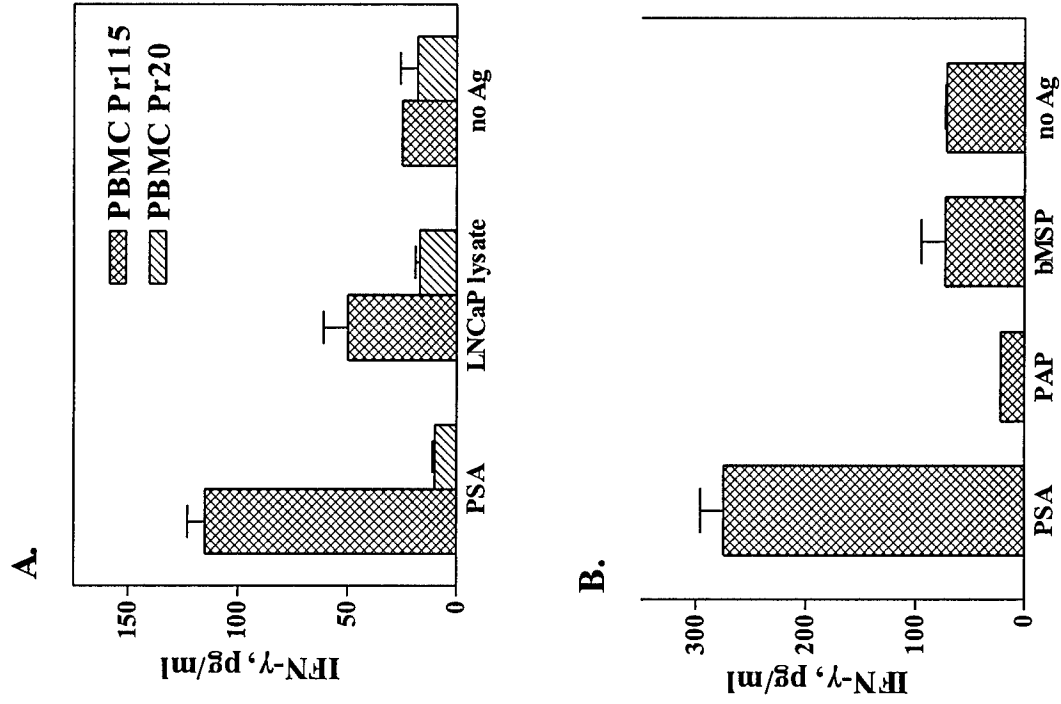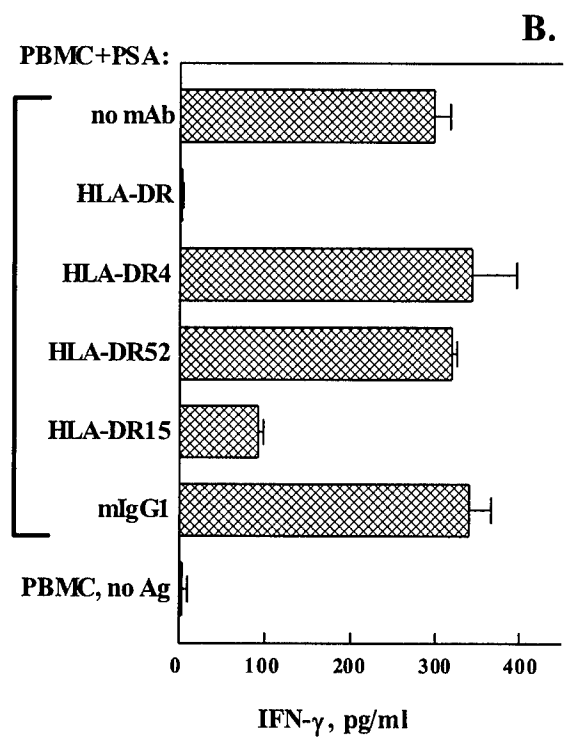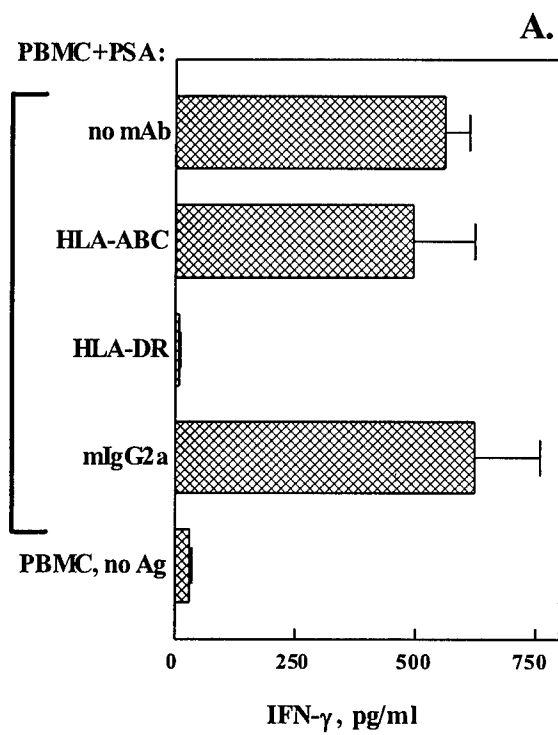
PSA
no Ag

IFN-γ, pg/ml

300
250
200
150
100
50
0

T-Pr90   T-Pr115

Responder

B. Anti-CD3

anti-CD3

2000
1750
1500
1250
1000
750
500
250
0

T-Pr90   T-Pr115

Responder

Figure 2

Figure 3

Figure 4
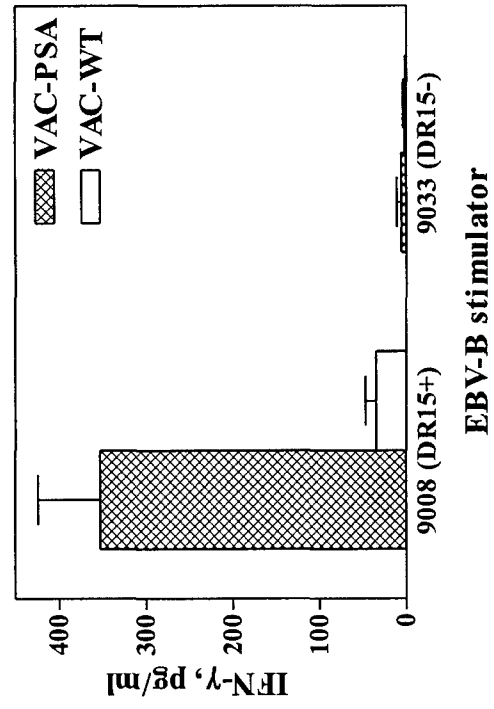
Figure 5
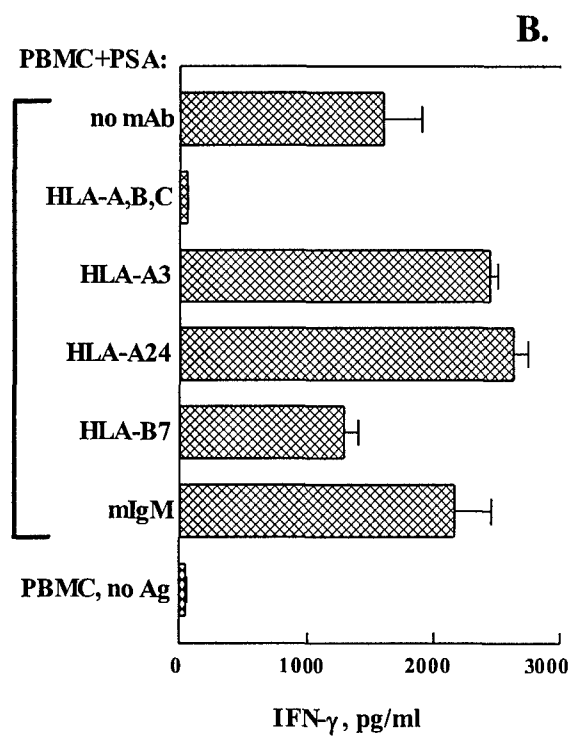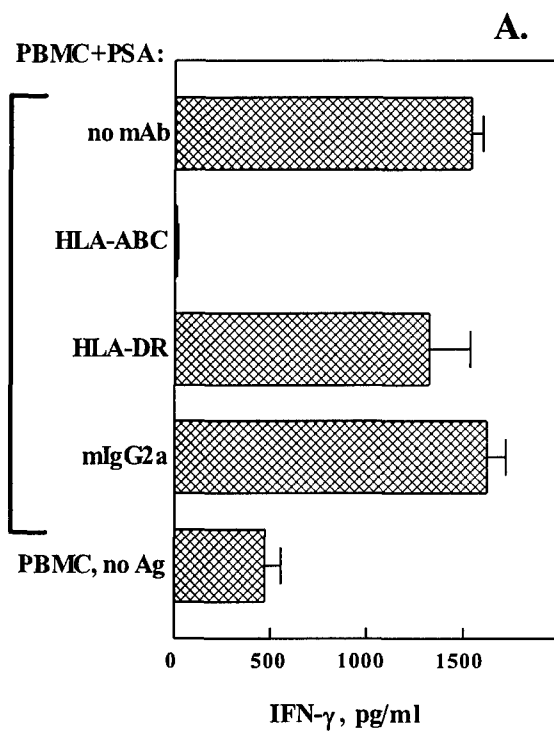
Figure 6
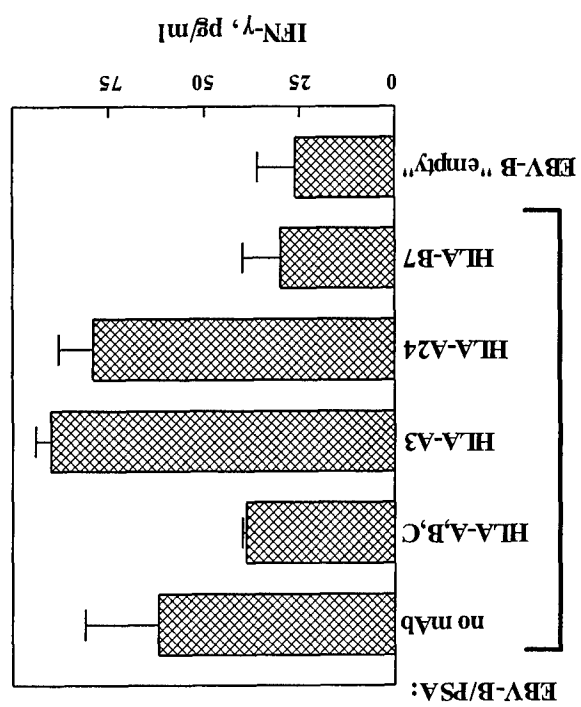
Abstract Title: **Generation of prostate specific antigen (PSA)-reactive T cell lines - implications for prostate cancer immunotherapy**

Abstract Author(s): E.N.Klyushnenkova, S. Ponniah, D.L.Mann, and R.B.Alexander

Abstract Institution(s): University of Maryland, Baltimore, MD

Abstract Body: One of the approaches to cancer immunotherapy is the induction of autoreactivity to differentiation antigens shared by tumor cells and normal cells of the same lineage. We have previously demonstrated that some patients with chronic prostatitis may have an autoimmune component to their disease because they have circulating T cells recognizing normal prostatic proteins and such cells were not found in normal men. To characterize this phenomenon further, we stimulated PBMC from such patients with purified PSA obtained from seminal fluid. After 14 days of culture, the cells were re-stimulated with PSA in the presence of irradiated autologous PBMC and IFN-γ secretion in the culture supernatants was measured by ELISA. T cells from 3 of 5 patients tested showed a specific and significant increase in IFN-γ secretion in response to PSA in secondary cultures. Several T cell clones derived from these cultures by limiting dilution retained their PSA reactivity as measured by increase in IFN-γ secretion. The PSA-specific responses of 4 of these clones were blocked specifically by anti-HLA-DR mAb while 3 other clones showed specific inhibition by anti-HLA-ABC mAb. Currently we are undertaking the characterization of fine specificity and MHC restriction of these clones. The data demonstrate that PSA-reactive CD4+ and CD8+ T cells can be derived from some patients with chronic prostatitis consistent with an autoimmune etiology of the disease. Since most prostate cancer cells continue to express PSA, immunization with PSA-derived peptides that are recognized by autoreactive T cells can be used as potential vaccines to induce anti-tumor reactivity in prostate cancer patients of appropriate HLA type.

Society for Basic Urologic Research
Fall Symposium
Sanibel Harbour Resort & Spa
November 9 – 12, 2000

Abstract Deadline: August 15, 2000

GENERATION OF HUMAN CD4 AND CD8 T LYMPHOCYTE LINES THAT RECOGNIZE PROSTATE SPECIFIC ANTIGEN (PSA) FROM PERIPHERAL BLOOD OF A PATIENT WITH GRANULOMATOUS PROSTATITIS. E. N. Klyushnenkova, S. Ponniah, A. Rodriguez, J. Kodak, D. L. Mann, and R. B. Alexander. University of Maryland, School of Medicine, Baltimore, MD

We have previously demonstrated that some patients with chronic prostatitis may have an autoimmune component to their disease because they showed a specific recall T cell proliferative response to PSA that was absent in normal donors. In order to demonstrate directly that PSA is a T cell antigen we generated long-term CD4+ and CD8+ T cell lines from peripheral blood mononuclear cells (PBMC) of a patient with granulomatous prostatitis using purified PSA as an antigen and recombinant IL-2 and IL-7. The HLA type of the patient was HLA-A*0301, A*2402, B*0702, B*1501, Cw*0303, Cw*0702, DRβ1*04, DRβ1*1501, DRβ4*01, DRβ5*0101. Several T cell clones derived by limiting dilution were specific for PSA as measured by at least a five-fold increase in IFN-γ secretion in response to PSA presented by irradiated autologous PBMC compared to unpulsed PBMC. Three clones were predominantly CD4+ whereas the other three clones were CD8+ as determined by flow cytometry. The PSA-specific responses of CD4+ clones were blocked by anti-HLA-DR mAb while CD8+ clones showed specific inhibition by anti-HLA-ABC mAb. IFN-γ secretion in response to PSA by all CD4+ T cell lines was blocked by antibody against HLA-DR15, but not HLA-DR4. The response of all CD8+ T cell lines was blocked by antibody against HLA-B7, but not HLA-A3 or HLA-A24. To confirm the specificity and HLA-restriction of PSA-specific T cell lines, we prepared EBV-B cell lines from HLA-matching donors that expressed recombinant PSA endogenously. EBV-B cell lines were transduced with a retroviral vector containing the PSA gene or were infected with a recombinant PSA-expressing vaccinia virus, and the expression of PSA was monitored by ELISA. CD4+ T cell lines secreted IFN-γ in response to HLA-DR15+ EBV-B cell lines that expressed PSA, whereas CD8+ T cell lines responded to HLA-B7+ PSA-expressing lines. HLA-matched targets transduced by control vectors as well as HLA-mismatched PSA-expressing targets did not induce the responses. Our data demonstrate that PSA-reactive CD4+ and CD8+ T cells can be derived from a patient with granulomatous prostatitis consistent with an autoimmune etiology of the disease. Our results extend the number of class I and class II alleles that can present PSA to generate CD8+ and CD4+ T cell responses. Since most prostate cancer cells continue to express PSA, immunization with PSA-derived peptides that are recognized by autoreactive T cells can be used as potential vaccines to induce anti-tumor reactivity in prostate cancer patients of appropriate HLA type.

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Name of person presenting abstract: _____

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Instructions: (1) Please type abstract single-spaced and within border. (2) Title should be typed in uppercase letters; do not list academic degrees. Type author names, institution, city, and country in upper and lowercase letters. Leave no margins. (3) Send original abstract and 3 copies to: SBUR Fall 2000. Department of Urology. 3125 RCP. University of Iowa. 200 Hawkins Drive. Iowa City. IA 52242-1089. (4) Deadline for submission is August 15. 2000. For more information. contact Linda Buckner at (319) 356-4275 or by fax at (319) 335-6971.

THERAPEUTIC ANTIBODIES FOR PROSTATE CANCER

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ABSTRACT

Twenty-five years ago, monoclonal antibodies were envisioned as magic bullets capable of targeting radioisotopes, toxins or cytotoxic drugs to the tumor site. It was soon realized that the potential of therapeutic antibodies far exceeded their use as carrier molecules and that native antibodies could also act as effector molecules capable of triggering a wide variety of anti-tumor responses. Today, we recognize that the utility and versatility of antibody-based products are unlimited; at the same time we have also learned that many obstacles need to be addressed to make antibody therapy an effective treatment modality. Past experiences from clinical trials and new development in the field of antibody technology have paved the way towards the creation of new strategies capable of circumventing or minimize these difficulties. Antibody-therapy is currently being tested in multiple trials for a variety of cancers including prostate cancer. This review discusses the different antibody-based strategies currently under investigation for prostate cancer.

INTRODUCTION

Prostate cancer is the most common invasive malignancy and second leading cause of cancer deaths in United States males [1]. Although locally confined disease is curable, 20% to 40% of patients develop recurrent disease after surgery or radiation therapy. Metastatic prostate cancer responds initially to androgen withdrawal therapy, but hormone resistance always develops and the majority of patients inevitably progress to

incurable, androgen-independent disease [2]. According to the American Cancer Society, an estimated 179,300 new cases will be diagnosed in 1999 and 37,000 men will die from the disease each year. Given the profound medical impact of prostate cancer and the lack of adequate therapies, there is a need to develop new modalities of treatment.

Our understanding of prostate cancer biology has grown tremendously over the last decade. This increase in knowledge has been accompanied by an impressive proliferation of new therapies ranging from new combinations of traditional therapeutic agents to novel agents intended to interfere with multiple aspects of prostate cancer progression. For example, novel approaches being tested in early clinical trials include immunotherapy, anti-angiogenesis therapy, differentiation therapy, and gene therapy [3]. This review will focus only on novel therapies employing antibodies as therapeutic agents. We do not intend to provide a complete inventory of the different strategies being studied, but rather to provide a general overview of the approaches under development.

MONOCLONAL ANTIBODIES FOR CANCER THERAPY

Monoclonal antibodies (mab) with their potential for diagnosis and therapy have marked a new era in the management of cancer. The transition of mab from laboratory reagents to clinical diagnostic agents was rapid and led to the development of invaluable assays for the detection and monitoring of many types of cancer. The development of therapeutic antibodies was, as expected, more challenging and their transition to the clinic much slower. The recent approval by the Food and Drug Administration (FDA) of two

recombinant mab for the treatment of cancer has generated considerable enthusiasm for antibody therapy. The first of these, Rituxan (IDEC Pharmaceuticals Inc.) approved in 1997 for non-Hodgkin's lymphoma, is a chimeric antibody directed against the CD20 antigen present on normal and malignant B-lymphocytes [4]. Trastuzumab or Herceptin (Genentech) was approved in 1998 for breast cancer. This unconjugated mab is directed against the product of the proto-oncogene HER2/neu that is overexpressed by one-third of breast cancer patients [5]. In addition to cancer therapy, mab have also been approved for cancer imaging and in other clinical settings such as transplantation (to abrogate rejection), and cardiovascular disease (to prevent clotting of vascular stents). Today more than 100 mab are being developed around the world as potential anti-cancer agents. More details on antibody therapy can be found in the following recent reviews [6, 7].

Despite the tremendous progress made in the field of immunology and cancer biology, we still know little about how mab operate *in vivo*. There are essentially 2 types of therapeutic antibodies used for cancer treatment: conjugated mab that are used as a vehicle for the delivery of cytotoxic agents, and mab that are themselves therapeutically active. The use of mab to target cytotoxic drugs to tumor cells was for many years the most popular approach to antibody-based therapy. In this context, antibodies that bind antigens on tumor cell surfaces have been linked to an agent which is therapeutically active such as toxins, drugs or radioisotopes. Use of unconjugated mab on the other hand, depends on the ability of the mab itself either to kill cells directly (*e.g.*, deliver an apoptotic signal) or to elicit an anti-tumor biological response. For example therapeutic antibodies can be designed to trigger an anti-tumor immune response, suppress tumor blood supply, or directly act on molecules necessary for tumor growth.

With advances in molecular biology technology, it is possible today to construct recombinant antibodies with wide variations in size, configuration, valence, and effector capabilities. As a result, antibodies with multiple effector functions can be generated allowing the development of novel treatment strategies. Such techniques also permit the production of therapeutic antibodies with minimal immunogenicity [8].

If we consider the clinical characteristics of cancer as one of the important criteria in the creation of effective strategies, several aspects of prostate cancer support the development of antibody-based therapies. In many cases, therapeutic approaches are frequently limited by expression of the targeted antigen on normal as well as malignant cells. Prostate cancer expresses two of the most specific tumor markers known in cancer biology: prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA). Antibodies with high affinity and specificity can specifically target these antigens. Other aspects in favor of antibody therapy for prostate cancer are the small volume of metastatic lesions and their accessibility to circulating antibodies. For instance, prostate cancer metastasizes predominantly to bone marrow and lymph nodes; these sites have been effectively treated by antibodies in other settings (*e.g.*, breast cancer and lymphoma). Finally, the maintenance of the quality of life of patients is an important point to consider, especially for slowly progressing diseases such as prostate cancer. Results from clinical trials have demonstrated that the injection of unconjugated antibodies is safe, with only minimal side effects.

PROSTATE TUMOR ANTIGENS FOR ANTIBODY THERAPY

Prostate Specific Antigen (PSA)

PSA (or hK3) is a 33 kDa serine protease belonging to the kallikrein family. This protein is primarily produced by the prostatic epithelium and is secreted into the seminal plasma where it can be found in high concentration. PSA is also detected in low levels in the sera of healthy males without clinical evidence of prostate cancer. However, with prostate malignancy, circulating levels of this antigen increase markedly, correlating with the clinical stage of the disease. PSA is now the most widely used marker for prostate cancer and is today regarded as the best tumor marker available. Virtually all primary (87-100%) and metastatic (94-100%) prostatic carcinomas stained positively with anti-PSA antibodies [9]. However, the intensity of the staining decreases in poorly differentiated primary tumors and in metastases [10]. PSA is neither tumor nor organ specific and has been found to be present in a number of female tissues and body fluids. For example, ultrasensitive assays for measuring PSA protein and mRNA have demonstrated that this protein is present in breast tissues and breast secretions, endometrium, amniotic fluid; sweat, periurethral and anal glands; and tumors of colon, lung, ovary, liver, kidney, adrenal, and salivary glands [11].

Prostate Specific Membrane Antigen (PSMA)

PSMA is a 100 kDa type II transmembrane protein produced primarily by the prostate gland. A high proportion of primary (90-100%) and metastatic (50-98%) prostate cancer expresses PSMA [12-14] and the cellular expression of this protein is increased in high-grade and hormone insensitive prostate cancer [15, 16]. In addition to the prostatic

epithelium, endothelial cells lining the capillary bed of a variety of tumors show positive immunoreactivity for PSMA [13, 17]. This observation suggests that PSMA could also be used for targeting the tumor vasculature. Although PSMA is considered as an excellent marker for prostate cancer, the expression of this antigen is not restricted to the prostate gland. Small but significant levels of PSMA have been detected in salivary glands, brain, duodenal mucosa and proximal renal tubules [13, 18, 19]. Moreover, PSMA-like proteins (PSMA' or PSM') have recently been identified [20, 21]. The extraprostatic expression of PSMA-like proteins and the cross reactivity of PSMA antibodies with PSMA-like proteins is of concern for PSMA-antibody therapy. However slight structural differences between PSMA and PSMA-like proteins should allow the production of antibodies that specifically target PSMA.

TAG-72

TAG-72 is a high molecular weight glycoprotein related to the sialylated Tn antigen expressed on a range of human carcinomas including colorectal, gastric, pancreatic, ovarian, endometrial, breast, non-small cell lung, and prostate. The expression of TAG-72 was demonstrated in both primary and metastatic prostate cancer as well as in androgen dependent- and independent tumors. In general, the proportion of cells staining with the antibody and the intensity of staining are higher in the primary (80%) than metastatic lesions (17-50%) and higher for hormone naïve (84%) than for androgen-independent tumors (50%). High level of TAG-72 expression is also found in healthy tissues such as colon, stomach, pancreas, ovary and testis [22, 23].

Epidermal growth factor receptor (EGFr)

Epidermal Growth Factor Receptor (EGFr or C-erbB) is a transmembrane glycoprotein with specificity for either EGF or TGF-alpha. The EGF receptor system is important in normal cell proliferation, migration and differentiation, and deregulation of this system is commonly observed in human cancers. Overexpression of EGFr has been reported in a wide variety of tumors including breast, ovarian, lung, and squamous cell carcinomas, and has been associated with less favorable prognosis and inferior disease-free survival. Similarly, for prostate cancer, a correlation between overexpression of the EGFr and poor clinical prognosis has been suggested. EGFr has been detected in both primary and metastatic prostate cancer, however, the level of expression seems much lower compared to other type of cancers [24-26].

HER-2/neu (ErbB2)

Her-2/neu proto-oncogene is the second member of the EGFr family. This gene encodes a 185 kDa transmembrane glycoprotein receptor (p185^{HER2}) normally expressed at a low level in a number of secretory epithelial cells. Her-2/neu amplification has been found in many tumors, including breast, ovarian, lung, gastric, and oral cancers [27]. Despite numerous studies, the overexpression of Her-2/neu in prostate cancer remains controversial [23, 28-32]. *In vitro* studies with different prostate tumor cell lines provide strong evidence supporting the role of Her-2/neu in the progression of prostate cancer to the androgen-independent state [33, 34].

THERAPEUTIC ANTIBODIES FOR PROSTATE CANCER

Anti-PSA antibodies

In addition to its utility as a marker for diagnosis and monitoring of prostate cancer, PSA is also recognized as an excellent target for immunotherapeutic strategies. An important point to consider for antibody-based therapies directed against PSA is the fact that this antigen is a secreted protein; such strategies can consequently not rely on an ADCC (antibody-dependent cell cytotoxicity) or CDC (complement-dependent cytotoxicity) mechanisms. We believe that this fact does not detract from the development of effective therapeutic products. Indeed past experience from clinical trials indicates that in most cases the amount of antibody targeted to the tumor site is not sufficient to elicit an ADCC or CDC cytolytic activity that could be translated into therapeutic effect. In addition, with the progress made in the field of immunology, we begin to understand that antibodies act through multiple mechanisms of action and that the observed therapeutic effect may be independent of an ADCC or CDC mechanism.

In collaboration with the biopharmaceutical company AltaRex Corp., we have developed a new treatment modality for prostate cancer that uses as a therapeutic agent a murine monoclonal antibody directed against PSA (ProstaRex™). AltaRex proprietary antibody-based immunotherapy technology is designed to enhance the ability of the human immune system to produce its own anti-tumor response [35-40]. In 3 different animal models, we have demonstrated that this antibody can inhibit the growth of PSA expressing tumors (Dr. Leveugle, personal communication). Results from *in vivo* and *in vitro* experiments highlight the importance of immune complex formation and suggest

that the capture of PSA / ProstaRex[TM] complexes by antigen presenting cells (APCs) plays a major role in the induction of an autoimmune response against PSA-expressing cells. It is today well established that the capture of immune complexes by Fc-receptors on APCs results in efficient priming of T-cell responses both *in vitro* and *in vivo* [41]. The enhanced processing of antigens into peptides presented by MHC class II molecules and the efficient stimulation of CD4^{+}-cells has been demonstrated in several systems [42-44]. Of particular interest is the recent demonstration that immune complexes can also promote the maturation of dendritic cells and efficient MHC class I presentation of peptides from exogenous IgG-complexed antigens [45]. In accord with these observations, we observed a significant stimulation of both CD4^{+} and CD8^{+} responses against PSA after presentation of PSA / ProstaRex[TM] complexes to human dendritic cells (Dr. Mann, personal communication). The region recognized by ProstaRex[TM] resides between the amino acids 139-163 of the PSA molecule (Dr. Leveugle, personal communication). This region has elicited much attention since it contains several motifs for HLA class I molecules, and is implicated in the generation of cytotoxic T-lymphocytes capable to lyse prostate tumor cells [46-48]. We believe that the specificity of ProstaRex[TM] for the region 139-163 of PSA is of particular importance since the binding of ProstaRex[TM] to PSA may protect this domain from extensive (inappropriate) proteolytic cleavage and favor the generation of HLA-I peptides essential for the activation of cytotoxic T-lymphocytes. For instance, a clipped form of PSA at residues 145-146 has been described indicating the sensitivity of this site to proteolytic degradation [49]. The proteolysis of PSA between amino acids 145-146 will preclude the

generation of HLA-peptide 141-150 and therefore the stimulation of cytotoxic lymphocytes specific for this sequence.

In a different aspect, the binding of ProstaRex™ to the region 139-163 of the PSA antigen may also be beneficial by inhibiting the enzymatic activity of PSA. PSA is a serine protease with chymotrypsin-like activity. A complete inhibition of this activity by ProstaRex™ was observed *in vitro* (Dr. Leveugle, personal communication). The enzymatic activity of PSA has been implicated in the proteolytic cascade during prostate cancer invasion and metastasis. Blocking PSA proteolytic activity with PSA-specific mab resulted in a dose-dependent decrease in the invasion of LNCaP cells in a matrigel assay [50].

Finally we have demonstrated that the immunization of mice with ProstaRex™ induces the production of anti-anti-idiotypic antibodies (Ab3) [51]. Such antibodies are produced by the host itself in response to the immunization and have by definition the same specificity than the injected antibody (i.e. ProstaRex™) [52]. Because Ab3 antibodies share the same specificity with ProstaRex™ , it is expected that they will have the same anti-tumoral activity. The advantage of Ab3 antibodies relies in their very long circulating half-life (up to several months) and consequently their prolonged anti-tumoral effect. For example Ab3 antibodies may be of considerable help in priming T-cells through antigen-antibody complexes since an efficient induction of T-cell response requires a constant stimulation for a period of 2 to 4 weeks by activated APCs. Similarly, Ab3 antibodies may play a role in the maintenance of the inhibition of the PSA enzymatic activity and consequently in the inhibition of cancer invasion and metastasis. Ab3 antibodies as well as the injected ProstaRex™ could also accumulate at the tumor

site and form immune complexes. Tissue-deposited immune complexes crosslinking Fc-receptors on infiltrating immune effector cells (neutrophils and macrophages) may in turn cause the release of inflammatory cytokines, proteolytic enzymes and other toxic molecules involved in the induction of auto-immune responses [53].

Anti-PSA antibodies have also been used as carrier proteins for conjugated chemotherapeutic drugs. The intravenous injection of anti-PSA IgG conjugated to 5-fluoro-2-deoxyuridine could selectively inhibit cell proliferation and induce the death of LnCAP protstate tumor cells grown in nude mice. [54, 55].

Anti-PSMA antibodies

PSMA is highly expressed in prostate cancer and is currently the target of a number of diagnostic and therapeutic strategies. Moreover, as mentioned earlier, PSMA is also expressed by the tumor neovasculature [17] suggesting that anti-PSMA mab may act as a double-edged sword by simultaneously targeting tumor cells and newly formed blood vessels required for tumor growth.

Clinical trials have demonstrated that radioimmunoscintigraphy with anti-PSMA mab is particularly sensitive for detecting soft-tissue metastases and recently the FDA approved the ^{111}In-labeled anti-PSMA mab, capromab pendetide (7E11-C5.3, CYT-356, or ProstaScint; Cytogen Corp.) as an imaging agent for prostate cancer [56, 57]. This antibody binds to an intracellular epitope of PSMA. Therefore, it has been postulated that efficient imaging is due to the binding of the radiolabeled antibody to dead cells and/or

cellular debris in metastatic lesions. Based on the success obtained with this antibody as an imaging agent, clinical trials testing the therapeutic efficacy of the ^{90}Y-CYT-356 have been initiated [58]. In these trials myelosupression was the dose-limiting toxicity and more recent protocols have now included EDTA, a chelating agent that removes free Yttrium from the circulation [59]. Because CYT-356 binds only to an intracellular site of the PSMA molecule, new anti-PSMA mab, directed against the extracellular domain of PSMA have been produced. One of these murine antibodies, ^{131}I-muJ591, is currently under clinical evaluation in a phase I study for patients with hormone independent prostate cancer [60].

A fully human bispecific antibody that targets PSMA and the Fc-receptor for IgA (FcαR1, CD89) expressed by cytotoxic effector cells has been produced by chemical conjugation of the Fab fragments of anti-PSMA '8C12' and anti-CD89 '14.1' respectively (Medarex Inc.). Preclinical studies show that this bispecific antibody can induce antibody-dependent cell cytotoxicity and monocyte-derived macrophage phagocytosis of PSMA-expressing cells [61].

Anti-TAG-72 antibodies

The anti-TAG-72 mab CC49 is being tested in clinical trials for diagnosis and therapy of a variety of carcinomas. In patients with androgen-independent or metastatic prostate cancer, interferon-γ or interferon-α were used in combination with ^{131}I-CC49 mab to enhance TAG-72 expression by tumor cells. Although the addition of interferon enhances the tumor uptake of radiolabeled CC49, only modest anti-tumor effects were observed in phase II studies [62, 63]. The murine origin of CC49 and the rapid development of a

human anti-mouse antibody response (HAMA) precluded multiple injections of CC49. Humanized and CH2-deleted derivatives of CC49 have recently been produced. Those constructs with their expected low immunogenicity may show promise for improved CC49 mab therapy [64-67].

Antibodies directed against growth factors (Anti-EGFr and anti-Her-2/neu antibodies)

Epidermal growth factor receptors are important mediators of cell growth, differentiation and survival. Two members of this family, the EGF receptor and Her-2/neu have been extensively studied as potential target for antibody based therapy. Results from animal experiments have demonstrated the importance of EGFr and Her-2/neu in the progression of prostate cancer, suggesting that antibodies directed against these growth factor receptors may be beneficial for the treatment of metastatic prostate cancer [24, 25, 34]. Antibodies directed against EGF receptor (C225 and ABX-EGF) and Her-2/neu (Trastuzumab) are currently being evaluated in phase I or I/II trials for prostate cancer. Both the chimeric mab C225 (Cetuximab, Imclone) and the human IgG2 mab ABX-EGF (Abgenix Inc.) block ligand binding and receptor activation *in vitro*, and demonstrate pronounced anti-tumor activity in animal models [68, 69]. The humanized antibody Trastuzumab (Herceptin, Genentech) has already proven its clinical utility in treating breast cancer patients. In a prostate tumor animal models Trastuzumab slowed the growth of androgen-dependent tumors. This effect was more pronounced if Trastuzumab was used in combination with paclitaxel and was observed in both androgen-dependent and independent tumors [70].

The recombinant immunotoxin AR209 is composed of an anti-Her-2/neu single chain antibody coupled to a portion of the *Pseudomonas* exotoxin-A. The evaluation of AR209 therapeutic efficacy on human prostate xenografts in nude mice shows that the immunotoxin could slow the progression of small tumors (<200 mm^3) [71].

MDX-H210 (Medarex) is a bispecific antibody which targets the Her-2/neu tumor antigen and FcγR1 (CD64) on neutrophils and mononuclear phagocytes. MDX-H210 is comprised of the F(ab)' of anti-Her-2/neu murine mab '520C9' chemically conjugated to the F(ab)' of anti-CD64 humanized antibody 'H22'. In Phase II clinical studies for late stage prostate cancer patients, MDX-H210 has shown quality-of-life improvements and reductions of PSA levels in some patients. In this clinical trial subcutaneous GM-CSF is combined with intravenous MDX-H210 in an attempt to improve the immunologic and anti-cancer activity of the antibody [72].

Antibody mediated tumor necrosis therapy

Cotara™ (Techniclone Corp.) is a chimeric mab which binds to DNA or DNA associated proteins and targets dead/decaying cells found in the core of solid tumors. The ^{131}I-labeled mab is used to irradiate the living cells of the tumor 'from the inside'; this approach is known as Tumor Necrosis Therapy (TNT). Cotara™ is presently in Phase II clinical trials in the U.S. for malignant glioma and Phase a I/II clinical trial in Mexico City for treatment of pancreatic, prostrate and liver cancers. Antibodies have poor tissue penetration and the route of administration of Cotara™ may be crucial for efficient anti-tumor effect. Both intratumoral and intravenous injections will be performed in the phase I/II trial.

Anti-VEGF antibodies

Tumor growth and metastasis are critically dependent on angiogenesis and therapies designed to inhibit the process of new blood vessel formation represents a promising new modality for the treatment of solid tumors. The importance of vascular endothelial growth factor (VEGF) in the angiogenic process is well established and antibodies capable of inhibiting VEGF from binding to its receptors have demonstrated their potential therapeutic utility in animal models bearing different types of tumors. For example in a prostate tumor model, VEGF-neutralizing antibodies were shown to be efficient in slowing the growth and metastatic dissemination of the DU-145 prostate tumor cells [73, 74]. A humanized monoclonal antibody directed against VEGF (RhuMAB, Genentech) is currently under evaluation in a phase II clinical trial for hormone refractory prostate cancer patients.

Anti-CTLA-4 antibodies

In addition to their use as targeting agents for tumor cells and tumor vasculature, therapeutic antibodies have also been used as a powerful tool to target immune cells and potentate anti-tumor immune responses. The CTLA-4 receptor is a critical inhibitory regulator of T-cell functions, and antibody-mediated blockade of CTLA-4 prevents T-cell downregulation and enhances T-cell-responses. Administration of an anti-CTLA-4 antibody to mice injected with the TRAMPC1 prostate tumor cell line, showed significant inhibition of tumor growth to complete rejection of the induced prostate cancer [75]. Adjunct CTLA-4 blockade could also considerably reduce metastatic relapse if administrated immediately after resection of an established TRAMPC2 prostate tumor [76]. When used in combination with a tumor cell vaccine, CTLA-4 blockade was also

effective in reducing tumor incidence in the TRAMP transgenic murine model [77]. A phase I clinical trial of anti-CTLA-4 antibody in patients with advanced prostate cancer is in progress.

CONCLUSION

A number of antibody-based products are currently being tested as potential therapeutic agents for prostate cancer and many of them have already reached phase I and/or II clinical trials. Several antibodies under investigation such as those directed against TAG-72, EGFr and Her-2/neu have already been tested in more advanced clinical settings for other types of cancers [5, 63, 78, 79]. The advanced stage in the clinic as well as the promising results obtained in other types of cancers represent definite advantages for these antibodies. The therapeutic efficacy of such antibodies in prostate cancer will however be dependent of the level of expression of the targeted antigens by prostate cancer cells, an issue that remains controversial at this time.

Other approaches under investigation employ anti-PSA or PSMA antibodies and take advantage of the very high specificity of these antigens for the prostatic tissue. The restricted expression of a targeted antigen to the tumor cells is expected to greatly limit toxicity to healthy tissues and for this reason, the search for "highly specific" tumor antigens has been a challenge taken by many scientists and a major effort of genomic program. PSA is often described as "the best tumor antigen discovered so far", and the more recent discovery of PSMA has also generated much enthusiasm for the

development of prostate cancer targeted therapies. However, antibody-targeted therapies have to deal with the non-specific uptake of a large amount of the injected antibody by immune cells through the Fc-receptors. When antibodies are used as carrier molecules and conjugated to cytotoxic agents, unwanted side effects such as immunosuppression may occur. This effect could be overcome by using single chain antibodies or Fab fragments. On the other hand strategies have been designed to take advantage of the recognition of the Fc-portion of the antibodies by immune cells [53]. Fc-directed therapy has the objective to induce an anti-tumoral immune response by targeting tumor antigens to specialized immune cells and/or by attracting immune cells to the tumor site. Fc-directed therapy can use native antibodies, and can be optimized with the production of recombinant antibodies with improved Fc-binding capacity, or bifunctional antibodies with specificity for Fc-receptor.

Poor tumor penetration by the injected antibodies has also been for long time a major limitation for tumor-targeted therapies. Novel strategies circumvent this problem by targeting the tumor vasculature (anti-VEGF mab, anti-PSMA mab) or specific populations of immune cells (anti-CTLA-4 mab, and in some instances Fc-receptor directed therapies) rather than the tumor itself.

Progress over the last two decades has been substantial, and antibody-based therapy has now shown its promise for patients with breast cancer and B-cell lymphoma. We may expect that in the near future antibody therapy will emerge as a viable treatment option for prostate cancer.

ACKNOWLEDGEMENTS

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THERAPEUTIC METHOD AND COMPOSITION UTILIZING ANTIGEN-ANTIBODY COMPLEXATION AND PRESENTATION BY DENDRITIC CELLS

(Attorney Docket No. 107823.186)

5   ## CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 60/203,635 filed on May 11, 2000; U.S. Provisional Application Serial No. 60/253,956 filed November 28, 2000; and U.S. Provisional Application Serial No. 60/253,671 filed November 28, 2000 the entire contents of each of which are fully incorporated herein by

10   reference.

BACKGROUND OF THE INVENTION

Field of the invention

The invention relates to immunotherapy. More particularly, the invention relates

15   to the use of antigen presenting cells, in particular dendritic cells, in immunotherapy.

Summary of the related art

T lymphocytes (*i.e.*, T cells), unlike B lymphocytes (*i.e.*, B cells), typically recognize their target antigen only when the antigen is presented in the context of the

20   major histocompatibility complex (MHC). Thus, to present antigen to T lymphocytes, which include T helper cells and cytotoxic T cells, the antigen must be presented in context of an MHC molecule on the surface of an antigen presenting cell.

In particular, one type of antigen presenting cell, dendritic cells, has recently become of interest in the area of cancer immunotherapy. Steinman, Annu. Rev. Immunol.

25   9: 271-296 (1991) teaches that dendritic cells are rare leukocytes that originate in the bone marrow and can be found distributed throughout the body. Bjork, Clinical Immunology 92: 119-127 (1999) teaches that dendritic cells receive increasing attention due to their potential inclusion as biological adjuvants in tumor vaccines. Dendritic cells express several receptors for the Fc portion of immunoglobulin IgG, which mediate the

30   internalization of antigen-IgG complexes (ICs). In this capacity, dendritic cells are used

to present tumor antigens to T cells. Avigan, Blood Reviews 13: 51-64 (1999) teaches that several approaches have been adopted to directly load tumor antigens onto dendritic cells, including the pulsing of tumor peptides onto mature dendritic cells. Timmerman *et al.*, Annu. Rev. Med. 50: 507-529 (1999) teaches that isolated dendritic cells loaded with tumor antigen *ex vivo* and administered as a cellular vaccine have been found to induce protective and therapeutic anti-tumor immunity in experimental animals. European Patent No. EP0553244 describes an antigen/dual-specific binding agent complex for stimulating a response to the antigen, where the binding agent specifically binds both the antigen and a cell surface receptor on an antigen-presenting cell, but where binding of the binding agent to the cell surface receptor does not block the natural ligand for the receptor.

The mechanism of action for dendritic cell antigen presentation has also been explored. Coughlan *et al.*, Veterinary Immunology and Immunopathology 49: 321-330 (1996) discloses that antigen uptake by dendritic cells via Fcγ receptors results in functional augmentation of antigen presentation and T cell proliferation in an *in vitro* sheep system. Regnault *et al.*, J. Exp. Med. 189: 371-380 (1999) teaches that Fcγ receptors induce dendritic cell maturation and promote efficient MHC class I-restricted presentation of peptides from exogenous, immunoglobulin (Ig) complexed antigens in the mouse system.

Thus, there remains a need to discover methods for utilizing dendritic cells to treat human diseases. The promise of dendritic cell-based approaches to treat diseases, such as cancer, underscores the need to actually develop such approaches as effective therapeutic treatments.

BRIEF SUMMARY OF THE INVENTION

The invention provides a therapeutically effective dendritic cell-based approach to the treatment of diseases associated with an antigen. The methods according to the invention comprise combining *ex vivo* an antigen associated with a disease and a dendritic cell binding agent specific for the antigen, with or without a dendritic cell, to provide a composition, and administering the composition to a patient having a disease associated with the antigen, wherein the composition-administered patient receives a therapeutic benefit.

Accordingly, in a first aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient suffering from the disease a composition comprising an antigen associated with the disease and a dendritic cell binding agent specific for the antigen, wherein the antigen is complexed to the dendritic cell binding agent and wherein the patient administered the composition receives a therapeutic benefit. Preferably, the patient is human.

In a second aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient a composition comprising an antigen associated with the disease, a dendritic cell binding agent specific for the antigen, and a dendritic cell autologous to the patient, wherein the patient administered the composition receives a therapeutic benefit. Preferably, the patient is a human.

In a third aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient suffering from the disease a composition comprising a host anti-xenotypic antibody and a xenotypic antibody specific for the antigen associated with the disease, wherein the patient administered the composition receives a therapeutic benefit.

In a fourth aspect, the invention provides a therapeutic composition comprising a purified dendritic cell binding agent that is specific for an antigen associated with a disease and the antigen associated with the disease. In preferred embodiments, binding of the dendritic cell binding agent to a receptor on a dendritic cell blocks binding of a

natural ligand to the receptor. In certain embodiments of the fourth aspect of the invention, administration of the composition to a patient suffering from the disease provides the patient a therapeutic benefit. Preferably, the patient is a human. Preferably, the dendritic cell binding agent is an antibody.

5   In a fifth aspect, the invention provides a therapeutic composition comprising a purified dendritic cell binding agent that is specific for an antigen associated with a disease, a dendritic cell, and the antigen associated with the disease. In preferred embodiments, binding of the dendritic cell binding agent to a receptor on the dendritic cell blocks binding of a natural ligand to the receptor. In certain embodiments,

10   administration of the composition to a patient suffering from the disease provides the patient a therapeutic benefit, wherein the dendritic cell is autologous to the patient. Preferably, the patient is human

        In a sixth aspect, the invention provides a therapeutic composition comprising a purified xenotypic antibody that is specific for an antigen associated with a disease and a

15   host anti-xenotypic antibody. In certain embodiments, administration of the composition to a patient suffering from the disease provides the patient a therapeutic benefit, wherein the dendritic cell is autologous to the patient. Preferably, the patient is human

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BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the results of antigen binding studies with monocytes (blue bars) and immature dendritic cells (red bars). Figure 1A is a bar graph showing the percentage of positive events of monocytes or immature dendritic cells following incubation with 1000 U/ml FITC-labeled CA125 antigen and the indicated amount of Alt-2 antibody. Figure 1B is a bar graph showing the mean channel intensity of monocytes or immature dendritic cells following incubation with 1000 U/ml FITC-labeled CA125 antigen and the indicated amount of Alt-2 antibody.

Figure 2 is a bar graph showing the uptake of 1000 U/ml FITC-labeled CA125 antigen in the presence of 0, 0.2, or 0.4 µg/ml Alt-2 antibody (red bars) or control MOPC-21 antibody (yellow bars).

Figure 3A and 3B are bar graphs showing the percentage of positive events (Figure 3A) and mean channel intensity (Figure 3B) of monocytes (striped bars) or immature dendritic cells (solid bars) incubated with 1000 U/ml FITC-labeled CA125 antigen and the indicated amounts of murine Alt-2 (blue solid and striped bars) or chimeric Alt-2 (red solid and striped bars).

Figures 4A, 4B, and 4C are bar graphs showing the effect of complexation with HAMA (Figure 4A), specific antigen (Figures 4A and 4B), or both (Figure 4C) on antibody binding to dendritic cells. Figure 4A shows the binding of FITC-labeled anti-CA125 antibody, Alt-2, or FITC-labeled anti-PSA antibody, Al-6, to dendritic cells in the presence or absence antigen or in the presence or absence of HAMA. Figure 4B shows the binding of the FITC labeled Alt-2 in the presence of 8000 U/ml CA125 when the concentration of Alt-2 is 0, 0.313, 0.625, 1.25, and 2.5 µg/ml. Figure 4C show the binding of 1 µg/ml FITC labeled Alt-2 in the presence of 8000 U/ml CA125 with or without human anti-mouse antibody (HAMA) at 0, 0.33, 1, and 2 µg/ml.

Figures 5A and 5B are bar graphs showing the uptake of CA125- Alt-2 immune complexes in monocytes and immature dendritic cells in the presence (red solid and striped bars) and absence (blue solid and striped bars) of HAMA as measured by the percentage of positive events (Figure 5A) and mean channel intensity (Figure 5B) for monocytes (striped bars) or immature dendritic cells (solid bars).

Figure 6 is a bar graph showing the *in vitro* T cell activation (both CD4+ (white bars) and CD8+ T cells (black bars)) by dendritic cells "armed" by the indicated primary stimulation and restimulation as determined by the number of IFNγ producing cells per 10^6 cells.

Figure 7 is a bar graph showing IFN-γ release from T cells stimulated with DC loaded with CA125, Alt-2, or CA125/Alt-2 complex (*i.e.*, CA125/αCA125 complex) at the indicated concentrations for a seven day incubation period (round 1; blue bars) or for an initial seven days and an additional seven days with freshly loaded DCs (round 2; red bars).

Figures 8A and 8B are bar graphs showing intracellular IFN-γ production from CD4+ T cells (red bars) or CD8+ T cells (blue bars) stimulated with DC loaded with CA125, Alt-2, or CA125-Alt-2 complex (*i.e.*, CA125/αCA125 complex) at the indicated concentrations for a seven day incubation period (Figure 8A) or for an initial seven days and an additional seven days with freshly loaded DCs (Figure 8B).

Figure 9 is a bar graph showing the *in vitro* T cell activation (both CD4+ and CD8+ T cells) to peptides generated by dendritic cells "armed" by the indicated primary stimulation and restimulation. T cell activation was determined by the number of IFNγ producing cells per 10^6 cells.

Figures 10A and 10B show the rate of complexation of anti-CA125 antibody, Alt-2, to circulating CA125 following injection of Alt-2. Specifically, Figure 10A is a bar graph comparing the levels of CA125/aCA125 complex (black bars) compared to the level of free circulating CA125 (dotted bars). Figure 10B is a line graph showing the rate of complexation of anti-CA125 antibody, Alt-2, to circulating CA125 following injection of Alt-2 (left side of the graph), and the amount of free Alt-2 antibody (green circles) cleared following injection time, as measured in percentage of the injected dose (%ID). With time, the percentage of free circulating CA125 decreases as the amount of complexed CA125 increases.

Figures 11A, 11B, and C are graphs showing the relationship between CA125 antigen specific B cell (Figure 11A) and T cell (Figures 11B and 11C) responses, CA125 level, and patient survival. Specifically, Figure 11A shows the induction of humoral anti-

CA125 response (y-axis) as compared to the level of circulating CA125 antigen present at the time of injection of the anti-CA125 antibody, Alt-2 (x-axis). Figure 11B shows the induction of a cellular anti-CA125 response (y-axis) as compared to the level of circulating CA125 antigen (x-axis), comparing pre-injection of Alt-2 (open triangles) to post-injection of Alt-2 (closed triangles). Figure 11C is a scatter graph showing the stimulation index of T cells (CA125-specific T cell proliferation) in patient before (pre) or after (post) Alt-2 injection, where patients having greater than 105 U/ml CA125 had the greatest T cell response.

Figure 12 is a bar graph showing the multi-epitopic nature of the response from anti-CA125 antibodies produced by thirteen patients.

Figures 13A and 13B are survival curves of patients receiving injection of Alt-2. Specifically, Figure 13A is a survival curve showing an increased survival in patients who developed a 3x increase in anti-CA125 antibody response following injection of Alt-2 (dotted line) as compared to those with developed a less than 3x increase in anti-CA125 antibody response following injection of Alt-2 (solid line). Figure 13B is a survival curve showing an increased survival in patients who developed a CA125-specific T cell response following injection of Alt-2 (dotted line) as compared to those who did not develop a CA125-specific T cell response following injection (solid line).

Figures 14A-14B show the preferential presentation of antigen complexed with antibody by antigen presenting cells such as macrophages and B cells. Specifically, Figure 14A is a bar graph showing the level of T cell proliferation as measured by the Stimulation Index (SI) using macrophages as antigen-presenting cells following stimulation by the CA125, Alt-2, B27.1, mIgG1, CA125 plus Alt-2, CA125 plus B27.1, and CA125 plus mIgG1 at 0.1 μg/ml (black dots on white bars), 1 μg/ml ((white dots on black bars), and 10 μg/ml (solid black bars). Figure 14B is a bar graph showing the level of T cell stimulation as measured by the Stimulation Index (SI) using B cells as antigen-presenting cells following stimulation by the CA125, Alt-2, B27.1, mIgG1, CA125 plus Alt-2, CA125 plus B27.1, and CA125 plus mIgG1 at 0.1 μg/ml (black dots on white bars), 1 μg/ml ((white dots on black bars), and 10 μg/ml (solid black bars).

Figure 15 is a bar graph showing the different levels of human IgG in reconstituted SCID/bg mice.

Figure 16 is a bar graph showing the different tumor volumes in mice treated with control IgG1 (yellow bars), Alt-6 only (green bars), PSA only (blue bars), and the PSA/α-PSA immune complex (red bars).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to immunotherapy. More particularly, the invention relates to the use of antigen-presenting cells, in particular dendritic cells, in immunotherapy. The invention provides a therapeutically effective dendritic-based approach to the treatment of diseases associated with an antigen. The patents and publications cited herein reflect the level of skill in this field and are hereby incorporated by reference in their entirety to the same extent as if each was specifically and individually indicated to be incorporated by reference. In the case of any conflict between a cited reference and this specification, this specification shall prevail.

The invention provides methods for treating a patient having a disease associated with an antigen. The methods according to the invention comprise combining *ex vivo* the antigen associated with the disease with a dendritic cell binding agent specific for the antigen to provide a composition, and administering the composition to a patient suffering from the disease associated with the antigen, wherein the patient receives a therapeutic benefit.

Accordingly, in a first aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient suffering from the disease a composition of an antigen associated with the disease and a dendritic cell binding agent specific for the antigen, wherein the antigen is complexed to (*i.e.*, specifically bound by) the dendritic cell binding agent, and wherein the patient administered the composition receives a therapeutic benefit.

In certain embodiments, an antigen/dendritic cell binding agent complex may be formed, for example, by combining *ex vivo* the antigen and the dendritic cell binding agent. By "combining *ex vivo*" means bringing into physical proximity outside of the body.

In certain preferred embodiments, the patient is a human. In other embodiments, the patient is preferably a non-human mammal, particularly a laboratory animal. Preferred non-human patients of the invention include, without limitation, mice, rats,

rabbits, non-human primates (*e.g.*, chimpanzees, baboons, rhesus monkeys), dogs, cats, pigs, and armadillos.

The methods according to the invention are useful for therapeutically treating patients having a disease associated with an antigen. As used herein, the term "disease

5   associated with an antigen" means a condition in which signs or symptoms of illness in a majority of patients are present when the antigen is present in the patient's body at a certain concentration, but in which signs or symptoms of illness are absent or reduced when the antigen is absent from the patient's body or present in the patient's body at a lower concentration. "Signs or symptoms of illness" are clinically recognized

10   manifestations or indications of disease.

It will be appreciated that a "patient suffering from a disease associated with an antigen" of the invention may not yet be symptomatic for the disease. Accordingly, a patient with circulating BRCA-1 is a patient according to the invention even though that patient may not yet be symptomatic for breast cancer or other adenocarcinoma.

15   Some non-limiting examples of such antigens associated with a disease include the prostate specific antigen (associated with prostate cancer), BRCA-1 and BRCA-2 antigens (associated with many adenocarinomas, including breast cancer, lung cancer, and pancreatic cancer), CA125 (associated with ovarian cancer), aberrant myelin basic protein (associated with Alzheimer's disease), gp120 (associated with HIV infection and AIDS),

20   MUC-1 (associated with breast cancer), EBNA-1 (associated with Epstein Barr Virus infection), CA19.9 (associated with colorectal, stomach, and pancreatic cancers), and TAG-72 (associated with ovarian, stromal, and pancreatic cancers), p53 (associated with various cancers).

Thus, in certain preferred embodiments, the antigen is a tumor-associated antigen.

25   A "tumor associated antigen" is an antigen in the patient's body that is made by tumor cells, and which may be presented on the tumor surface, or circulating, or both. Preferred tumor-associated antigens include, without limitation, CA125, PSA, MUC-1, CA19.9, and TAG-72. Generally from about 0.1 to about 50 μg antigen are used.

In certain preferred embodiments, the antigen is from a pathogen. A "pathogen"

30   is an etiolytic agent capable of causing disease. Preferred pathogens include, without

limitation, viruses (*e.g.* hepatitis B, hepatitis C, herpes, and HIV-1), viroids, bacteria, fungi, prions, and parasites.

"Specifically bound to the antigen" or "specific for the antigen" means that the dendritic cell binding agent binds to the antigen with greater affinity than it binds unrelated proteins. Preferably such affinity is at least 10-fold greater, more preferably at least 100-fold greater, and most preferably at least 1000-fold greater than the affinity of the binding agent for unrelated proteins. Preferably, an antigen presenting cell binding agent that is specific for an antigen forms an association with that antigen with an affinity of at least $10^6 M^{-1}$, more preferably, at least $10^7 M^{-1}$, even more preferably, at least $10^8 M^{-1}$, even more preferably, at least $10^9 M^{-1}$, and most preferably, at least $10^{10} M^{-1}$ either in water, under physiological conditions, or under conditions which approximate physiological conditions with respect to ionic strength, *e.g.*, 140 mM NaCl, 5 mM MgCl$_2$.

The injected dendritic cell binding agent complexed to a circulating antigen is targeted *in vivo* to dendritic cells (which are preferably immature dendritic cell) through Fc receptors present on the surface of these dendritic cells. By targeting antigen to preferably immature dendritic cells and presentation of these antigens on both MHC class I and class II molecules, the immune complex of the binding agent/antigen efficiently sensitize dendritic cells to induce activation of both CD4(+) helper and CD8(+) cytotoxic T cells *in vivo*.

A "dendritic cell binding agent" of the invention binds to the ligand-binding site of a receptor on the surface of a dendritic cell, at any stage of development of the dendritic cell. Preferably, once the dendritic cell binding agent is bound to the ligand-binding site of the dendritic cell receptor, the natural ligand cannot bind to the receptor at the same time that the dendritic cell binding agent binds to the receptor. Preferably, the dendritic cell binding agent binds to the receptor on the surface of a dendritic cell when the binding agent is specifically bound to an antigen. Preferably, such binding causes internalization of the binding agent/antigen complex. Even more preferably, binding and/or internalization of the binding agent/antigen complex by an immature or precursor dendritic cell causes maturation and/or activation of the dendritic cell.

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Preferably, the dendritic cell binding agent of the invention binds to an activating Fcγ receptor, such as CD64 (FcγRI) or CD32 (FcγRIIA) that is not abundant on neutrophils.

As used herein, by "ligand-binding site of a receptor" is meant the site on the
5   receptor to which the natural ligand of the receptor binds. For example, if the receptor is a Fcγ type II receptor, the natural ligand for the receptor is an IgG antibody. A dendritic cell binding agent of the invention, when bound to a receptor, blocks the ligand-binding site of the receptor such that the natural ligand for that receptor cannot bind the receptor. In one non-limiting example, if the receptor is a Fcγ type II receptor and the dendritic cell
10   binding agent of the invention is an IgG antibody, then binding of the dendritic cell binding agent of the invention to the receptor prevents other IgG antibodies from binding to the receptor.

Accordingly, dendritic cell binding agents of the invention are readily identified by art-known methods. In one non-limiting example, where the dendritic cell binding
15   agent is an IgG antibody, a precursor, immature, or mature dendritic cell is purified as described below. Next, the cell is incubated with the FITC-labeled IgG antibody (with or without the antigen to which the antibody specifically binds). Next, the phycoerythrin (PE)-labeled natural ligand (i.e., another IgG antibody) is added to the cell. The cell can then be subjected to analysis by flow cytometry to determine if the FITC-labeled IgG
20   antibody of the invention is able to block binding of the PE-labeled antibody to the receptor on the dendritic cell.

In certain preferred embodiments, the dendritic cell binding agent is not a bispecific antibody which has two antigen binding sites, one that is specific for the antigen of the invention and the other that is specific for the receptor, e.g., at its ligand-
25   binding site, on the surface of a dendritic cell.

"Administering the composition to a patient" means providing the composition to the patient in a manner that results in the composition being inside the patient's body. Such an administration can be by any route including, without limitation, sub-cutaneoous, intradermal, intravenous, intra-arterial, intraperitoneal, and intramuscular. "Receives a
30   therapeutic benefit" means that the patient experiences alleviation or reduction of signs or

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symptoms of illness, and specifically includes, without limitation, prolongation of survival. In certain preferred embodiments of the methods according to the invention, a CD8+ IFN-γ producing T cell is activated to induce a cytotoxic T lymphocyte (CTL) immune response in the patient administered the composition. In certain embodiments of

5   the methods according to the invention, a CD4+ IFN-γ producing T cell is activated to induce a helper T cell immune response in the patient administered with the composition. These activated CD4+ IFN-γ producing T cells (*i.e.*, helper T cells) provide necessary immunological help (*e.g.*, by release of cytokines) to induce and maintain not only CTL, but also a humoral immune response mediated by B cells. Thus, in certain embodiments

10   of the methods according to the invention, a humoral response to the antigen is activated in the patient administered with the composition.

Activation of a CD8+ and/or CD4+ IFN-γ producing T cells means causing T cells that have the ability to produce IFN-γ to actually produce IFN-γ, or to increase their production of IFN-γ. "Induction of CTL" means causing potentially cytotoxic T

15   lymphocytes to exhibit antigen-specific cytotoxicity, or to increase such antigen-specific cytotoxocity. "Antigen-specific cytotoxicity" means cytotoxicity against a cell that is presenting the antigen that is greater than cytotoxicity against a cell that is not presenting the antigen. "Cytotoxicity" refers to the ability of the cytotoxic T lymphocyte to kill the target cell. Preferably, such antigen-specific cytotoxicity is at least 3-fold, more

20   preferably 10-fold greater, more preferably more than 100-fold greater than cytotoxicity against a cell that is not presenting the antigen.

In certain preferred embodiments, the dendritic cell binding agent of the invention binds to the antigen and an Fcγ type II or type I receptor on the dendritic cells. Preferably, binding of the binding agent to the Fcγ type II or type I receptor blocks the binding of the

25   natural ligand to, respectively, the Fcγ type II or type I receptor. Accordingly, in certain embodiments, the dendritic cell binding agent binds to the antigen and to an Fcγ Type I (CD64) receptor on a dendritic cell in the patient administered with the composition. In certain embodiments, the dendritic cell binding agent binds to the antigen and to an Fcγ Type II (CD32) receptor, such as a Fcγ Type IIA (CD32A) receptor or a Fcγ Type IIB

30   (CD32B) receptor on a dendritic cell in the patient administered with the composition. In

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certain embodiments, the dendritic cell binding agent binds to the antigen and to an Fcγ Type II CD16 (FcγRIII) receptor on a dendritic cell in the patient administered with the composition.

In certain preferred embodiments, the dendritic cell binding agent of the invention
5   is an antibody. Preferably the antibody is provided at a concentration of from about 1-10 μg/ml. An "antibody" includes a molecule comprising an active-portion of an antibody. "An active portion of an antibody" is a molecule that includes an antigen binding site that is specific for an antigen and a receptor binding site that binds an Fc receptor on its ligand-binding site (e.g., the Fc portion of the antibody included the heavy chain constant
10   region). Accordingly, an antibody of the invention may be, e.g., chimeric, single chain, mutant, or antibody fragment so long as the antibody is able to specifically bind an antigen and so long as the antibody includes a portion that binds an Fc receptor on its ligand-binding site.

Accordingly, an antibody of the may be encoded by an immunoglobulin gene
15   having specific point mutation in the part of the gene encoding the receptor binding site. In one non-limiting example, the gene encoding the anti-PSA antibody, Alt-6, can be subjected to point mutation in the portion of the gene encoding the receptor binding site. The resulting antibody mutants can be screened on cells (e.g., COS or HeLa cells) transfected with a gene encoding the Fcγ Type I (CD64) receptor, the Fcγ Type II (CD32)
20   receptor, or the Fcγ Type II CD16 (FcγRIII) receptor. A preferred mutant Alt-6 antibody of the invention is one which binds better (i.e., by greater numbers or with higher affinity) to a cell expressing the Fcγ Type I (CD64) receptor, Fcγ Type II (CD32) receptor and/or the Fcγ Type II CD16 (FcγRIII) receptor as compared to a cell not expressing one of these receptors.

25   In certain embodiments, the dendritic cell binding agent of the composition includes a portion that elicits a human anti-xenotypic antibody (HAXA) response.

In certain embodiments, the dendritic cell binding agent is a xenotypic antibody. A "xenotypic antibody" is an antibody from a species other than the patient's species. For example, if the patient is a human, a dendritic binding agent of the invention that is a
30   murine antibody is a xenotypic antibody. Similarly, if the patient is a mouse, a dendritic

binding agent of the invention that is a rat antibody is a xenotypic antibody. Preferred xenotypic antibodies include monoclonal antibodies, including without limitation murine monoclonal antibodies. Particularly preferred murine monoclonal antibodies include Alt-1 (murine IgG1, specifically binds to MUC-1; ATCC No. PTA-975; American Type Culture Collection, Manassas, VA), Alt-2 (murine IgG1, specifically binds to CA125; ATCC No. PTA-1883), Alt3 (murine IgG3, specifically binds to CA19.9; ATCC No. PTA-2691), Alt-4 (murine IgM, specifically binds to CA19.9; ATCC No. PTA-2692), Alt-5 (murine IgG1, specifically binds to CA19.9; ATCC No. PTA-2690); and Alt-6 (murine IgG1, specifically binds to prostate specific antigen (PSA); ATCC No. HB-12526).

In certain embodiments, the xenotypic antibody elicits a host anti-xenotypic antibody (HAXA) response in the patient.

In certain preferred embodiments, the composition administered to the patient further includes host anti-xenotypic antibodies (HAXA). "Host anti-xenotypic antibodies (HAXA)" are antibodies of the host animal species that bind to the xenotypic antibody contained in the composition of the invention. For example, if the patient is a human and the xenotypic antibodies are rabbit antibodies, the HAXA is human anti-rabbit antibodies. Preferably the HAXA is provided in the composition at a concentration of from about 1-10 μg/ml. Preferred HAXA include, without limitation, human anti-mouse antibody (HAMA).

In certain embodiments, if the composition administered to the patient does not include HAXA, HAXA is preferably already present in the patient's blood. Such a patient may already have HAXA if, for example, the patient has been previously treated with a non-specific antibody of the same species as the dendritic cell binding agent of the invention. For example, prior to the administration of the composition of the invention comprising the dendritic cell binding agent and the antigen, a human patient may be administered with a polyclonal murine antibody with no defined specificity (or with a murine monoclonal antibody that specifically binds to, for example, an egg shell protein not expressed in humans). Once HAMA is detectable in the patient's blood, the composition is administered to the patient.

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In further embodiments, the composition of the first aspect further comprises a dendritic cell. Preferably, the dendritic cell is autologous to the patient.

As used herein, by "dendritic cell" is meant a bone marrow-derived cell that can internalize antigen and process the antigen such that it (or a peptide derived from the antigen) is presented in the context of both the MHC class I complex and the MHC class II complex. Accordingly, a dendritic cell of the invention is able to activate both CD8+ T cells (which are primarily cytotoxic T lymphocytes) and CD4+ T cells (which are primarily helper T cells). It should be understood that any cell capable of presenting a peptide derived from an internalized antigen on both class I and class II MHC is a dendritic cell of the invention. Preferably, a dendritic cell of the invention has the phenotype and characteristics of the dendritic cells described in Steinman, Annu. Rev. Immunol. 9: 271-296 (1991).

In certain preferred embodiments, the dendritic cell, when added to the composition, is an immature dendritic cell. As used herein, by "immature dendritic cells" means a population of dendritic cells preferably having one or more of the following cell surface antigens at the indicated level of expression: CD11c present on greater than about 90% of the dendritic cells in the population, HLA-DR present on fewer than about 90% of the dendritic cells in the population but on greater than about 70% of the dendritic cells in the population, HLA-ABC present on from about 80% to about 90% of the dendritic cells in the population, CD14 present on fewer than about 20% of the dendritic cells in the population, CD16 present on greater than about 10% to about 40% of the dendritic cells in the population, CD80 present on about 50% to about 70% of the dendritic cells in the population, CD86 present on greater than about 40% to about 70% of the dendritic cells in the population, CD83 present on greater than about 10% to about 20% of the dendritic cells in the population, CD64 present on greater than about 40% to about 60% of the dendritic cells in the population, and CD32 antigen present on from about 70% to about 95% of the dendritic cells in the population, and about less than 10% of the dendritic cells in the population are CD3/CD19 positive (*i.e.*, about less than 10% have CD3/CD19 expression).

In certain preferred embodiment, the dendritic cell, when added to the composition, is a precursor dendritic cell. As used herein, by "precursor dendritic cells" means a population of cells, each of which is capable of becoming a dendritic cell, where greater than 80% of the population have CD64 and CD32 antigen present and about 70%

5   of the population is positive for CD14.

In the embodiments of the invention where the dendritic cell, when added to the composition, is either an immature dendritic cell or is a precursor dendritic cell, the composition is preferably incubated *ex vivo* under conditions (*e.g.*, in cell culture) that allow for maturation of the immature dendritic cell or precursor dendritic cell prior to

10   administering the composition to the patient. Such conditions that allow the formation of mature dendritic cells from immature or precursor dendritic cells are described below in the examples.

In the embodiments of the invention where the dendritic cell is included in the composition, and where the patient is human, the dendritic cell preferably expresses the

15   cell surface molecules described below in Table I at its different maturation stages. Note that expression of the Fc receptors, particularly the CD64 (FcγRI) typically decreases as the DC mature.

Table I
Human Dendritic Cell Surface Markers

	Day 0	Day 4	Day 7
Marker (all cells)	*Monocytes*	*immature DC*	*mature DC*
HLA-DR	70-85%	80-85%	95-99%
HLA-ABC	70-85%	85-90%	95-99%
CD3	1-5%	ND	ND
CD4	2-3%	ND	ND
CD8	2-3%	ND	ND
CD16	3-15%	15-40%	0.5-5%
CD19	5-10%	ND	ND
CD14	75-80%	0.4-0.5%	0.1-0.2%
CD11c	75-80%	95-99%	99-100%
Marker (gated on DC)			
CD86	85-90%	40-70%	95-99%
CD80	30-50%	55-80%	85-90%
CD40	40-50%	55-60%	55-60%
CD83	10-15%	10-15%	55-60%
CD32	89-98%	70-95%	40-45%
CD64	92-99%	28-60%	4-10%

One non-limiting method to obtain dendritic cells according to the invention is described below in Example I.

Accordingly, in one non-limiting method, 10 µg of CA125 and 5 µg of the murine monoclonal antibody that specifically binds to CA125, Alt-2, are combined together *ex vivo*. In a variation of the method, human anti-murine antibodies are added to the mixture. Then, the mixture is added to immature dendritic cells prepared as described below from the patient suffering from the disease. The addition of the antigen (in this case CA125 plus αCA125 antibody, Alt-2) promotes maturation of the immature dendritic cells. (Note that as used throughout, the symbol "α" means "anti-". Thus, "αCA125" means "anti-CA125"). Next, the matured dendritic cells "loaded" or "armed" with CA125 and Alt-2 (and, in some cases, HAMA) are removed from culture and administered to the patient.

Note that the dendritic cell used in the invention is preferably autologous to the patient to whom the composition of the invention is administered. By "autologous" is meant having identically matched MHC loci (both class I and class II). Thus, an identical sibling can provide autologous dendritic cells for a patient. Similarly, a close relative can provide autologous dendritic cells for a patient, so long as the patient and the close relative have identically matched MHC loci. Of course, two individuals of an inbred strain of laboratory animal (*e.g.*, inbred Balb/c mice) are autologous to one another.

In certain preferred embodiments, if the patient to whom the composition of the invention is administered already had an immune response to the antigen, following administration, the immune response is shifted predominantly from a helper to a cytotoxic T cell response, thus providing the patient, following administration, a therapeutic benefit

Thus, in one non-limiting example, a patient of the invention with prostate cancer may already have either antibodies that are specific for prostate specific antigen (PSA) and/or helper T cells that are specific for PSA. However, following administration of the composition of the invention, the PSA of the composition is internalized and presented on antigen presenting cells in such a way (*e.g.*, in context of MHC class I) that cytotoxic T cells that are specific for PSA are stimulated, thereby providing the patient a therapeutic benefit as compared to the patient's condition prior to administration of the composition.

In a second aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient a composition comprising an antigen associated with the disease, a dendritic cell binding agent specific for the antigen, and a dendritic cell autologous to the patient, wherein the patient administered the composition receives a therapeutic benefit. Preferably, the patient is a human

In some embodiments, the dendritic cell, when combined with the antigen and the dendritic cell binding agent, is either a precursor dendritic cell or an immature dendritic cell. In these embodiments, the composition is incubated *ex vivo* under conditions that allow for maturation of the immature dendritic cell prior to administering the composition to the patient.

In some embodiments, the antigen and dendritic cell binding agent may be combined simultaneously with the dendritic cell to make the composition. In some embodiments, the dendritic cell binding agent and the antigen are combined with one another before they are combined with the dendritic cell to make the final composition. In these embodiments, the composition including the dendritic cell is then administered to the patient suffering from the disease.

In certain embodiments, where the dendritic cell binding agent is a xenotypic antibody and the composition further comprises human anti-xenotypic antibodies.

In a third aspect, the invention provides a method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient suffering from the disease a composition comprising a host anti-xenotypic antibody and a xenotypic antibody specific for the antigen associated with the disease, wherein the patient administered the composition receives a therapeutic benefit.

In a fourth aspect, the invention provides a therapeutic composition comprising a purified dendritic cell binding agent that is specific for an antigen associated with a disease and the antigen associated with the disease. Preferably, binding of the dendritic cell binding agent to a receptor on a dendritic cell blocks binding of a natural ligand to the receptor.

In a fifth aspect, the invention provides a therapeutic composition comprising a purified dendritic cell binding agent that is specific for an antigen associated with a disease, a dendritic cell, and the antigen associated with the disease. Preferably, binding of the dendritic cell binding agent to a receptor on the dendritic cell blocks binding of a

5   natural ligand to the receptor. Preferably, the dendritic cell of the composition is autologous to a patient to whom the composition is administered.

In a sixth aspect, the invention provides a therapeutic composition comprising a purified xenotypic antibody that is specific for an antigen associated with a disease and a host anti-xenotypic antibody.

10   As used herein, by "purified" is meant that the indicated agent (*e.g.*, a purified dendritic cell binding agent or xenotypic antibody) has been separated from components which naturally accompany it. For example, in the case of a protein (*e.g.*, a dendritic cell binding agent), the purified protein is separated from components, such as other proteins or fragments of cell membrane, that accompany it in the cell. Of course, those of ordinary

15   skill in molecular biology will understand that water, buffers, and other small molecules may additionally be present in a purified protein preparation. A purified protein (*e.g.*, a purified dendritic cell binding agent) of the invention is at least 95% by weight, more preferably at least 98% by weight, even more preferably at least 99% by weight, and most preferably 100% by weight free of components which naturally accompany the nucleic

20   acid molecule or polypeptide.

According to the invention, a purified dendritic cell binding agent of the invention may be generated, for example, by recombinant expression of a nucleic acid molecule encoding the dendritic cell binding agent in a cell in which the dendritic cell binding agent does not naturally occur. Of course, other methods for obtaining a purified

25   dendritic cell binding agent of the invention include, without limitation, artificial synthesis of the dendritic cell binding agent on a peptide synthesizer and isolation of the dendritic cell binding agent from a cell in which it naturally occurs using, *e.g.*, an antibody that specifically binds to the dendritic cell binding agent. In the case where the dendritic cell binding agent is a monoclonal antibody, a purified dendritic cell binding

30   agent can be obtained from the culture supernatant of the hybridoma which secretes the

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dendritic cell binding agent, or from ascites fluid from an animal injected with the hybridoma.

Preferably, the therapeutic compositions of the fourth, fifth, and sixth aspects of the invention further comprise a pharmaceutically acceptable carrier. By "pharmaceutically acceptable carrier" is meant a carrier that is physiologically acceptable to the administered patient and that retains the therapeutic properties of the dendritic cell binding agent and antigen (and/or dendritic cell) with which is it administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other pharmaceutically-acceptable carriers and their formulations are well-known and generally described in, for example, Remington's Pharmaceutical Sciences (18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990).

Preferably, administration of the therapeutic compositions of the fourth, fifth, and sixth aspects of the invention to a patient suffering from the disease provides the patient a therapeutic benefit. Preferably, the patient is a human.

The following examples are intended to further illustrate certain particularly preferred embodiments of the invention and are not intended to limit the scope of the invention.

Example I
Isolation of Dendritic Cells

To isolate dendritic cells, peripheral blood mononuclear cells (PBMC) were isolated from the apheresis products from normal volunteers by ficoll-hypaque (Histopaque 1.077, commercially available from Sigma, St. Louis, MO) gradient centrifugation, and viably frozen using an automated cell freezer (commercially available from Gordinier Electronics, Roseville, MI) in RPMI (commercially available from Life Technologies, Frederick, MD) containing 40% human antibody serum (commercially available from Gemini Bio-Products, Woodland, CA) and 10% DMSO (Sigma). The cells were stored in the vapor phase of liquid nitrogen until used. DNA was prepared from a portion of the cells and used for molecular HLA typing.

Next, dendritic cells (DC) were isolated by negative selection. To do this, DC precursors were prepared from freshly-thawed PBMC by negative selection using immunomagnetic bead depletion. Specifically, PBMC were placed into a tube and incubated on ice for 30 min. with mouse anti-human CD3, CD16, and CD19 antigens

5   (commercially available from Caltag, Burlingame, CA). Excess antibody was removed by washing the cells with phosphate buffered saline containing 1% of bovine serum albumin (PBS/0.1% BSA), and the washed cells were next incubated with Pan Mouse IgG immunomagnetic beads (commercially available from Dynal, Lake Success, NY) for 30 min. on ice. The tube containing the cells plus specific mouse anti-human antigens

10   and the Pan Mouse IgG immunomagnetic beads was placed against a magnet to remove the cell:bead complexes. The cells that bound to the magnet were either T cells, B cells, or Natural Killer (NK) cells. Accordingly, the supernatant contained the lineage-depleted DC precursors (*i.e.*, the monocytes remaining in the fluid in the tube not expressing CD3, CD16, or CD19 antigens and so not bound by the magnet). These

15   negatively selected cells were approximately 70% pure monocytes as characterized by Flow cytometry using a broad CD marker panel (see Table I above) were collected.

Next, the negatively selected cells were washed, resuspended in cRPMI (RPMI supplemented with 1% glutamine and 10% heat-inactivated human serum (from a person with blood type AB)) containing GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) (both

20   commercially available from R & D Systems, Minneapolis, MN) and cultured at 37°C in 5% CO_2 at 0.5 x 10^6 cells/well in 24 well plates for four days. These cells were immature dendritic cells by day four, and analyzed for surface expression of numerous cell surface antigens by flow cytometry see Table I above).

On the fourth day of culture, the cells were pulsed with antigen (*e.g.*, Prostate

25   Specific Antigen (PSA)) and incubated for an additional three days. (Note that the antigen which the DC cells were pulsed with was dependent upon what the DC cells would be eventually used for. For example, if the DC cells were to be used to generate a T cell response to PSA, the DC cells would be pulsed with PSA antigen.) Several agents which are known to cause DC precursors to mature into mature DC cells were added to

30   the cultures eight hours after addition of the antigen. These agents included TNFα (10

µg/ml) and/or IFNα (50 µg/ml). The matured DC were harvested on the seventh day of culture, analyzed for phenotypic markers by flow cytometry and used in functional studies.

To analyze DC for cell surface marker expression by flow cytometry, standard

5 methods were employed. Briefly, the cells were aliquotted into polystyrene tubes and stained for surface markers with fluorochrome-labeled murine antibodies. The complete DC cell surface marker panel included: HLA-A, HLA-A B, HLA-A C, HLA-DR, CD14, CD11c, CD123, CD4, CD40, CD83, CD86, CD80, CD16, CD32, CD64 (specific detectably labeled antibodies to which are commercially available from Becton

10 Dickinson, San Jose, CA). Following a 30 min. incubation on ice, the cells were washed with PBS and pelleted by centrifugation. The cell pellets were resuspended in 250 µl of fixative (2% paraformaldehyde). The data was acquired using a FACSCan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with Cellquest software (Becton-Dickinson, San Jose, CA).

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Example II
Phenotypic Markers on Dendritic Cells

Initial studies were focused on the uptake of CA125 in monocytes and dendritic cells in the presence or absence of Alt-2. For this purpose, CA125 was purified from

20 tissue culture supernatant of NIH:OVCAR-3 cells (AltaRex Corp.). Highly purified CA125 was labeled with Fluorescein (Flourescein-EX, Molecular Probes), and incubated with monocytes, immature dendritic cells, and mature dendritic cells under various conditions. In some experiments, the uptake of MAb-Alt-2 was also followed, where the MAb-Alt-2 was labeled with Fluorescein-EX or Cy-3.

25 As shown above in Table I, monocytes, immature DC, and mature DC could be easily distinguished based on their expression of various cell surface antigens.

30

Example III

Comparison of CA125 Antigen Uptake in Monocytes to Immature Dendritic Cells

In these studies, highly purified CA125 was labeled with Fluorescein and incubated with monocytes or immature dendritic cells at 1000 U/mL for 1 hour at 37°C.

5   In some cases, unlabeled antibody was added simultaneously with the labeled antigen to study the effect of complex formation on the uptake by the two antigen-presenting cells. The binding to cells was quantified by flow cytometry and the results are shown in Figures 1A (percent positive events) and 1B (mean channel intensity). As shown in Figures 1A and 1B, immature dendritic cells showed much higher uptake of the antigen,

10   with MAb Alt-2 as compared to without MAb Alt-2. Both monocytes and immature dendritic cells showed an increase of CA125 uptake in the presence of MAb Alt-2 (see Figures 1A and 1B, respectively). For the immature dendritic cells, the CA125 concentration needed to be lowered to 1000 U/mL to allow the detection of an antibody effect (concentration below the saturation point for CA125 uptake).

15   It is interesting to note that immature DCs showed an increase in the number of cells capable of taking up the CA125 antigen (Figure 1A, % positive cells) as well as the concentration of antigen within each cell (Figure 1B, mean fluorescence intensity) with increasing concentrations of MAb Alt-2. In monocytes, increasing antibody concentrations could only enhance the percentage of cells capable of taking up the CA125

20   antigen. These results may indicate that the immune complexes are either taken up by different receptors in monocytes and immature DCs or that the immune complexes are taken up by the same receptors in monocytes and immature DCs, but the receptor is recycled at a higher frequency in immature DCs. The receptor is certainly more abundant and/or is internalized more rapidly in immature DCs than monocytes, demonstrated by

25   the higher percentage of targeted cells in DCs (see Figure 1A).

Next, the requirement for antibody specificity was tested. To do this, highly purified CA125 was labeled with Fluorescein and incubated with immature dendritic cells at 1000 U/mL for 1 hour at 37°C in the absence and presence of unlabeled MAb Alt-2 or MOPC-21 (a control murine IgG1 that does not bind to the CA125 antigen). The binding

30   to cells was assessed by flow cytometry, and the results are shown in Figure 2. As Figure

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2 shows, antibody-enhanced uptake of CA125 was specific for MAb Alt-2 and could not be achieved with a control antibody that does not bind to CA125. Consequently, the antibody-enhanced uptake is due to a more efficient uptake route in DCs and not due to stimulation of the endocytotic activity of dendritic cells upon receptor engagement by the binding MOPC-21 antibody

Example IV
Effect of Murine Alt-2 and Chimeric Alt-2 on CA125 Antigen Uptake

Other binding studies have compared the uptake of CA125 in the presence of a murine and humanized form of MAb Alt-2. In this study, fluorescein-labeled CA125 was incubated with monocytes and immature DCs at 1000 U/mL for 1 h at 37°C in the presence of murine (mAlt-2) and chimeric Alt-2 (cAlt-2 (chimeric with a human IgG3 constant region)). The binding to cells was assessed by flow cytometry.

As shown in Figures 3A and 3B, the chimeric antibody showed slightly better enhancement of CA125 binding to monocytes than the murine Alt-2; on immature dendritic cells, the murine and chimeric antibody were equally effective. As for the murine antibody, the chimeric Alt-2 increased the percentage of CA125-targeted cells within the monocytes (Figure 3A) and dendritic cell populations, but increased the amount of CA125 per cell only in dendritic cells (Figure 3B).

Example V
Effect of HAMA on CA125 Antigen Uptake

Complexation of antibody with specific antigen or with HAMA and binding to dendritic cells was measured. To do this, human anti-mouse antibody (HAMA) was purified from patient serum samples with high HAMA concentrations after MAb Alt-2 injection via affinity chromatography on Protein G, and a MAb-AR20.5 column, followed by negative selection on a MAb Alt-2 column to eliminate Ab2 (*i.e.*, human antibody that binds to the idiotype of the MAb Alt-2 antibody).

Five micrograms (5 μg) FITC-labeled Alt2 (anti-CA125) or Alt-6 (anti-PSA) murine monoclonal antibody was incubated together with the corresponding antigen (1

μg) and/or HAMA (2.5 μg) and 5 x 10^5 dendritic cells at 37°C for 60 minutes. The mixture was then washed once and resuspended in 0.5% formalin + PBS and subjected to FACScan on a FACSCalibur machine (Beckton-Dickinson). The results for Alt-2 and CA125 are shown in Figures 4A, 4B, and 4C. These results demonstrate that binding of antibody to dendritic cells is enhanced by complexation of antibody to its antigen, complexation of antibody with HAMA, or complexation of antibody to its antigen in the presence of HAMA.

Further binding studies were conducted to compare the uptake of the CA125-MAb-Alt-2 complex by monocytes or immature dendritic cells in the presence and absence of HAMA. In this study, fluorescein-labeled CA125 + murine MAb-Alt-2 was incubated at 1000 U/ml of CA125 and a range of MAb-Alt-2 concentrations with monocytes or immature dendritic cells for 1 hour at 37°C. The binding studies were conducted in the absence and presence of human anti-mouse antibodies (HAMA). The HAMA concentrations were equivalent to the MAb-Alt-2 concentrations to form equimolar complexes of HAMA and MAb-Alt-2. The binding to cells was assessed by flow cytometry.

As shown in Figures 5A (percent positive cells) and 5B (mean fluorescence intensity), HAMA further enhanced the binding of CA125 to human monocytes and dendritic cells. Immune complexes with HAMA increased the percentage of CA125-targeted cells within the monocytes and dendritic cell populations (Figure 5A), but increased the CA125 concentration per cell mainly in dendritic cells (Figure 5B).

Example VI
Alt-6 Antibody and Alt-6-PSA Immune Complex Binding to DC at Different Stages of Development and Maturation

DC of different stages of development and maturation were first analyzed by flow cytometry to determine their level of expression of Fcγ receptor. To do this, DC were isolated from PBMC as described above and tested for Fcγ receptor expression with fluorescence labeled antibodies at day 0, after 4 days in culture with GM-CSF/IL-4 and after maturation with TNFα and IFNα (7 days). Binding was determined on CD11c+,

HLA-DR+ cells and is expressed in Table II (below) as percentage of positive cells within the gate.

As shown below on Table II, DC express Fcγ type I, and type II receptors (CD64 and CD32), and low levels of FcγR III (CD16)

Table II
Distribution of Fcγ Receptors on DC at Different Stages of Maturation

	Days in culture		
Fcγ Receptor	0	4	7
	Positive Cells [%] (gated on DC)		
CD16 (FcγRIII)	3 – 15	15 – 40	0.5 - 5
CD32 (FcγRII)	89 – 98	70 – 95	40 - 45
CD64 (FcγRI)	92 – 99	28 – 60	4 - 10

The percent of DC expressing these Fcγ receptors differed depending on their stage of maturation/differentiation. As shown on Table II, the number of DC expressing CD16 was highest after culture for 4 days with GM-CSF/IL-4 and was nearly absent on DC after maturation (*i.e.*, after 7 days in culture with GM-CSF/IL-4). CD32 and CD64 were found on the majority of DC precursors. CD32 was expressed on 70-95% of immature DC and declined to about 40% on mature DC. In addition, the number of CD64 expressing cells declined as the DC differentiated and matured.

Next, to determine if the anti-PSA monoclonal antibody Alt-6 and Alt-6-PSA immune complexes would bind to DC and if the stage of DC differentiation and maturation affected binding, a range of concentrations of the fluorescein-labeled Alt-6 as well as Alt-6-PSA-FITC-complexes were added to three different populations of DC: I) freshly isolated myloid DC precursors (surface phenotype-CD11c+, HLA-DR+, CD14+), II) DC after culture for 4 days in GM-CSF/IL-4 (surface phenotype-CD11c+, HLA-DR+, CD14-, CD86+) and III) DC that had been further matured by culturing for an additional 3 days with TNFα and IFNα (surface phenotype-CD11c+, HLA-DR+, CD86+, CD40+, CD80+, CD83+) (see Table I above).

To do this, PSA was purchased from Scripps Laboratories (San Diego, CA) and the murine monoclonal anti-PSA antibody Alt-6 (ProstaRex™) was kindly provided by

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AltaRex Corporation, Edmonton, Alberta, Canada (ATCC No. HB-12526; American Type Culture Collection, Manassas, VA). Alt-6 is an IgG1 antibody that reacts specifically with an epitope that maps to the region of amino acid residues 137-146 (sequence EPEEFLTPKK) of PSA. PSA and Alt-6 were diluted in cRPMI to

5   concentrations of 5 and 25 μg/ml, respectively. When mixed at these concentrations, approximate equimolar amounts of PSA and anti-PSA were achieved.

Alt-6 was labeled with Fluorescein-EX (commercially available from Molecular Probes, Eugene, OR) following manufacturers instructions. Equimolar concentrations of PSA were mixed with 1.25 μg/ml, 2.5 μg/ml, 5.0 μg/ml, and 25 μg/ml of Alt-6-FITC and

10   added to freshly isolated DC precursors, to DC that had been cultured for 4 days in the presence of GM-CSF/ IL-4 and to DC that had been matured.

The antibody-PSA composition was incubated for 1 hour at 37°C, washed, pelleted, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. DC were gated in the sideward/forward scatter based on DC preparations stained in parallel with the

15   complete DC marker panel (see Example I). The percentage of cells that reacted with Alt-6 and Alt-6-PSA complexes were calculated based on binding to CD11c+, HLA-DR+ cells. Table III (below) shows percentage of positive cells within the gate.

Table III
20   PSA-Specific Monoclonal Antibody Alt-6 and Alt-6-PSA Immune Complex Binding to DC at Different Stages of Maturation

		Days in culture		
		0	4	7
	Antibody Concentration	Positive Cells (gated on DC) [%]		
Alt-6-FITC	1.25 μg/ml	0.13	0.68	
	2.5 μg/ml	0.16	1.56	
	5 μg/ml	1.84	9.95	1.19
	25 μg/ml	7.33	28.1	
Alt-6-FITC + PSA	1.25 μg/ml	1.82	2.41	
	2.5 μg/ml	3.91	8.70	
	5 μg/ml	5.38	23.40	1.56
	25 μg/ml	18.00	72.11	

As shown in Table III, at the concentrations tested, Alt-6 bound to a low percentage of freshly isolated DC precursors. The number of cells binding the antibody increased after culture for 4 days in GM-CSF and IL-4. Interestingly, the number of cells binding Alt-6-FITC was significantly higher in the presence of PSA, indicating that the Fc receptors have enhanced binding to immune complexed antibody over the antibody alone, or that immune complexes bind to different and/or more abundant receptors on DC precursors and immature DC. No binding of the Alt-6 or the immune complexes occurred to mature DC.

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Example VII

T Lymphocyte Responses to DC armed with PSA or PSA/anti-PSA

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T lymphocytes were stimulated with DC armed with PSA or a combination of PSA/anti-PSA antibody.

To do this, monocytes were generated from leukaphoresis samples from healthy donors (Biological Specialty Corp., Colmar, PA) and depleted from lineage cells by incubation with anti-CD3, CD19 and CD 16 antibodies, followed by incubation with magnetic bead conjugated anti-mouse Ig and separation on a magnet (Dynal). Negatively selected cells were approximately 70% pure monocytes as characterized by flow cytometry using a broad CD marker panel (see Example I). Monocytes were incubated with IL-4 and GM-CSF (R&D Systems) for 4 days in RPMI1640 + 10% matched human serum to generate immature DC. Again, an aliquot of the cells was stained with a broad CD marker panel to ensure purity and identity of the cells. These cells were then harvested and loaded with antigen combinations (e.g., PSA, the anti-PSA monoclonal antibody and the antibody-PSA combination) for 2 - 8 hours at 37°C, and then matured with IFN-α and TNF-α for three days. Dendritic cell were checked again via flow cytometry for an array of CD markers to ensure proper maturation of the cells.

Specifically, the immature DCs were loaded with PSA (25 μg/mL), Alt-6 (5 μg/mL) and PSA + Alt-6 (25 μg/mL of PSA; 5 μg/mL of Alt-6). These immature DCs were added to T cells that were generated from the same monocytes as the DCs via negative selection (i.e., autologous T cells), using a magnetic T cell isolation kit (commercially available from Dynal). Briefly, to isolate T cells, CD3+ T lymphocytes

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were isolated from thawed PBMC (see Example I) by negative selection (using magnetic beads commercially available from Dynal, Lake Success, NY). Briefly, the cells were incubated on ice for 30 min with a mixture of antibodies to CD14, CD16, CD56 and HLA Class II DR/DP. Excess antibodies were removed by washing with PBS/0.1% BSA. The

5   cells were incubated for 30 min. at room temperature with immunomagnetic beads coated with an anti-mouse IgG antibody (Dynal). The cells were placed against a magnet and the T lymphocytes, which did not bind the magnet (*i.e*, did not express CD14, CD16, CD56, and HLA class II DR/DP) were isolated from the supernatant.

        T cells and DCs were incubated for 7 days (primary stimulation), re-stimulated

10   with loaded and matured DCs and incubated for another 7 days. For stimulation, T lymphocytes were plated in twenty-four well plates at a concentration of 1×10^6 cells/well and to which were added 5×10^4 DC that were antigen naive or that had been exposed to PSA, the anti-PSA monoclonal antibody or the combination of PSA and the monoclonal antibody. An aliquot of the cells was taken 24 hour later and prepared for

15   intracellular cytokine staining (Day 15; primary stimulation), whereas the remaining cells were incubated for another 7 days (secondary stimulation) and prepared for intracellular cytokine staining on Day 22. Prior to adding the second or third DC preparation, an aliquot of the cell supernatant was taken for testing in an IFN-γ ELISA (Pharmingen DouSet).

20   For the intracellular cytokine staining, cells were incubated with Golgi Plug (R&D Systems) 2 hours after DC addition and incubated for another 16-18 h. Cells were stained with anti-CD3-FITC and anti-CD8-Cy-Chrome for 30 min. on ice, washed, permeabilized, and stained with anti-IFN-γ-PE for 30 min. on ice. Cells were washed, fixed and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

25   T cell responses were measured as numbers of CD4+ and CD8+ T cells producing intracellular IFNγ. To do this, flow cytometry was used. Briefly, Brefeldin A (10 μg/ml) (commercially available from Pharmingen, San Diego, CA) or Golgi Plug (commercially available from R&D Systems) was added to the T cell cultures 2 hours after restimulation with antigen armed DC. After an additional 16-18 hours of culture, cells were stained

30   with anti-CD3-FITC and anti-CD8-Cytochrome for 30 min. on ice, washed,

permeabilized (*e.g.*, by incubating in a perm/fix solution (Pharmingen) for 20 min. on ice), and stained with anti-IFN-γ-PE for 30 min. on ice in staining buffer (PBS with 1% human AB serum (*i.e.*, human sera from a person with type AB blood) (antibodies commercially available from Becton Dickinson, San Jose, CA) added. The cells were

5   washed, resuspended in staining buffer containing 2% paraformaldehyde and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

In this experiment, mature "armed" or "un-armed" DC (i.e., matured with cytokines alone) were co-cultured with autologous T cells, restimulated with armed or un-armed DC, and the number of CD4+ and CD8+ cells producing IFNγ was determined.

10   The results of a representative experiment are shown in Figure 6.

As shown in Figure 6, little to no difference in number of IFNγ producing CD4+ or CD8+ T cells were found in co-cultures with DC that were not exposed to antigen (negative control) or T cells cultured with DC that had been exposed to the monoclonal antibody (*i.e.*, α-PSA as primary stimulated) and were then restimulated with armed or

15   un-armed DC. In all combinations of primary stimulation and restimulation, PSA armed DC stimulated CD4+ T cell responses were consistently greater than CD8+ T cell responses. Notably, strong CD8+ T cell responses were observed on restimulation with immune complex armed DC (*i.e.*, where restimuation was with the complex and primary stimulation was with the complex of PSA/α-PSA). When T cells were cultured with

20   antigen-antibody armed DC (*i.e.*, primary stimulation is PSA/αPSA) and restimulated with PSA armed DC, CD4+ T cell responses were substantially greater than CD8+ responses.

On the other hand, consistent CD4+ and CD8+ IFNγ responses were only generated in T cells exposed to and restimulated by antigen-antibody armed DC (*i.e.*,

25   where both primary stimulation and restimulation was PSA/α-PSA). Since IFNγ T cell responses were not generated to the Alt-6 antibody (*i.e.*, α-PSA alone as primary stimulation or restimulation), it is expected that the response to the complex is directed at the PSA. The increase in CD8+ T cell responses to PSA presented in combination with the antibody compared to the responses with free PSA alone indicate that the immune

30   complex enhances antigen processing, in particular, through the HLA Class I pathways.

Example VIII

T Lymphocyte Responses to DC Armed With CA125 or CA125/αCA125 Complex

After identifying broad ranges of functional CA125 and MAb- Alt-2
concentrations for T cell stimulation in small checkerboard assay for IFN-γ release into
5   the supernatant, intracellular cytokine staining assays were performed using 50, 500 and
5000 U/mL of CA125 in the absence and presence of 2.5 μg of MAb-Alt-2. Matured DC
cells and T cells were incubated for 7 days and then re-stimulated with loaded and
matured DCs. Seven days after stimulation with loaded DCs (Figure 7, round 1), and
after the restimulation (Figure 7, round 2), an aliquot of the cell supernatant was taken
10   and tested in an IFN-γ ELISA (Pharmingen DouSet).

As a positive control, PMA + Ionomycin was used for stimulation of the T cells in
culture. DCs loaded in medium only were used to determine the background of the assay.

As shown on Figure 7, stimulation with CA125 alone did not result in substantial
IFN-γ release, detected by IFN-γ ELISA of the cell supernatants (Figure 7).

15   For intracellular staining studies, immature DCs were loaded with CA125 (50,
500, 5000 U/mL), Alt-2 (2.5 μg/mL) and CA125 + Alt-2 (50, 500, 5000 U/mL of CA125;
2.5 μg/ml of Alt-2) and matured. T cells and DCs were incubated for 7 days, and re-
stimulated with loaded and matured DCs. An aliquot of the cells was taken 24 hours later
and prepared for intracellular cytokine staining (Day 15; Figure 8A), whereas the
20   remaining cells were incubated for another 7 days. Those cells were stimulated with
another batch of loaded and matured DCs and prepared for intracellular cytokine staining
on Day 22 (Figure 8B). For the intracellular cytokine staining, cells were incubated with
Golgi Plug (R&D Systems) 2 hours after DC addition and incubated for another 16-18
hours. Cells were stained with anti-CD3-FITC and anti-CD8-Cy-Chrome for 30 min. on
25   ice, washed, permeabilized, and stained with anti-IFN-γ-PE for 30 min. on ice. Cells were
washed, fixed and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

As shown on Figures 7, 8A, and 8B, responses to CA125 could be detected only
after two stimulation rounds and at the highest concentration tested. As shown in Figures
8A and 8B, antigen alone mainly stimulated CD4 responses as shown by the intracellular
30   staining results. As shown on Figure 7, substantially increased IFN-γ release was

observed in responses to CA125 presented in a complex with MAb-Alt-2 by human DC.
IFN-γ release into the cell supernatant, detected by IFN-γ ELISA, as well as intracellular
cytokine production (ICC) could be detected already after one stimulation round and was
further increased after the second round of stimulation (Figures 7 and 8B, respectively).
CA125 concentrations as low as 50 U/mL were able to stimulate T cells, with an
optimum of 500 U/mL at two stimulation rounds (Figures 7, 8A, and 8B).

Of particular note is the finding that the CA125-MAb Alt-2 immune complexes
induced initially strong CD4 responses (Figure 8A) and with additional stimulation also
strong CD8 responses (Figure 8B). These observations indicate that CA125 can be
presented on MHC class I and II if presented in the form of an immune complex, but only
on MHC class II molecules, if taken up alone, as expected for extracellular antigens. The
receptor responsible for the uptake of the antigen or antibody-antigen complex may
influence the route for processing of the internalized protein. Antigen-antibody
complexes may have increased leakage into the cytosol and therefore better chance of
being processed by the proteasomes, the processing machinery that transports peptides
onto MHC class I molecules via the invariant chain.

Example IX
PSA/anti-PSA Complex-Armed DC Generate HLA-A*0201 Peptide-Specific T
Lymphocyte Responses

The ability of PSA/anti-PSA immune complexes and PSA armed DC to generate
T lymphocyte responses to two PSA peptides that are known to be restricted by the HLA-
A*0201 allele was next compared. To do this, two HLA-A2 specific PSA peptides
(pep1, FLTPKKLQCV; pep2, KLQCVDLHV) and an HIV-1 peptide (SYNTVAVL)
were synthesized by the Biopolymer Laboratory, University of Maryland, Baltimore, MD,
diluted in cRPMI, added to the DC preparations at concentrations of 5 µg/ml, and
incubated for approximately 1 hour.

DC were prepared as described in Example I and exposed to PSA or PSA/anti-
PSA complex after 4 days of culture in GM-CSF/IL-4. Following maturation with
CD40L or TNFα and IFNα, the armed DC were cultured with autologous CD3+ selected

T lymphocytes for 7 days. The T cells were next harvested, newly armed DC added to the cell culture, and the cells cultured for an additional 7 days in media supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml) (see Example III).

At day 14, the T cells were harvested and restimulated with matured DC that were
5   pulsed with the two PSA-derived peptides and analyzed for IFNγ release.

Results of a representative experiment (1 of 3) are shown in Figure 9. In T cells cultured with PSA-armed DC for 2 weeks, low levels of CD4+ responses were detected upon restimulation with the PSA-derived peptides. Peptide 2 (KLQCVDLHV) also induced small CD8+ responses when the primary stimulation was PSA alone. Consistent
10   with the previous results described in Example III, restimulation of T cells with PSA-armed DC (where the primary stimulation was PSA) resulted in CD4+ T cell responses, and low levels of responding CD8+ T cells. Restimulation with the PSA/anti-PSA complex (where the primary stimulation was PSA) showed similar activation of CD4+ as well as CD8+ INFγ responses.

15   As the lower half of Figure 9 shows, T cells cultured with PSA/anti-PSA armed DC for two weeks were able to respond to both PSA-derived peptides. Peptide-restricted responses were approximately twice as strong in T cells cultured with PSA/anti-PSA armed DC before restimulation as compared to the responses to peptide by PSA-armed DC (*i.e.*, T cells whose primary stimulation was PSA alone).

20   Note that T cells propagated in the presence of PSA-armed DC were able to respond weakly to peptide 2 (KLQCVDLHV), but the CD8+ T cells did not release IFNγ upon restimulation with PSA-armed DC. This results suggests that this peptide may be presented by HLA Class II molecules as well as by Class I, but that the generation of T cells specific for this peptide are not effectively generated by DC loaded with PSA. No
25   responses were found when the T cells that had been cultured with immune complex were stimulated with mature DC that had been pulsed with an HLA-A*0201 restricted HIV-1 gag peptide (data not shown).

30

Example X

T cell responses to DC armed with PSA or PSA-m

DC express receptors that bind mannose and related carbohydrates. Accordingly, experiments were conducted to compare the T cell responses to DC that had been armed with PSA or PSA that had been conjugated to mannose. To do this, PSA was purchased from Scripps Laboratories (San Diego, CA). PSA was mannosylated as follows: 100 μg of PSA was combined with 100 μg α-D-mannopyranosylphenylisothiocyanate (a-D-M) and 2 μl N-methylmorpholine in 460 μl of PBS and stirred overnight at room temperature. Excess α-D-M was hydrolyzed by the addition of 100 μl of 1M Trizma-base (pH 9.5). Unconjugated mannose residues were removed by dialysis against PBS and stored at 4°C.

The T cells were exposed through two weekly rounds of stimulation to DC armed with PSA or PSA-m and CD4+ and CD8+ IFNγ responses measured after re-exposure to armed DC. To do this, as described in Example III, T cells were exposed to DC armed with PSA or mannosylated-PSA (PSA-M) and cultured. At day 14, T cells were restimulated with unarmed or antigen armed DC and numbers of CD4+ and CD8+ T cells producing IFNγ determined by flow cytometry. The results are shown in Table IV.

Table IV
Comparison of T Cell Responses to Dendritic Cells Armed With PSA or Mannosylated PSA

		IFNγ+ cells/10^6	
Primary stimulation	Restimulation	CD4+	CD8+
O	PSA	33	42
PSA	O	109	41
PSA	PSA	438	88
PSA	PSA-M	566	92
O	PSA-M	80	61
PSA-M	O	111	82
PSA-M	PSA-M	894	122
PSA-M	PSA	504	113

As show in Table IV, in three separate experiments, CD4+ T cell responses predominated within the activated cell population and were consistently higher when DC

were loaded with PSA-m compared to responses to PSA armed DC. Small to modest increases (over controls) in number of activated CD8+ T cells were observed. However, the CD8+ T cell response to PSA-m was far below responses seen with Alt-6-PSA immune complexes (see Figure 6).

5

Example XI
Clinical Studies

Patients suffering from recurring ovarian cancer were injected with 2 mg monoclonal antibody Alt2 per day, with a total of 1 to 10 such treatments and followed

10   for disease progression and survival. Alt2 is a murine monoclonal IgG1 antibody to CA125. The antibody has a high affinity ($1 \times 10^{10} M^{-1}$) and was modified, such as by photoactivation (partial reduction of disulfide bonds, see U.S. Patent No. 6,086,873). All patients were tested for HAMA and CA125 before and after each injection by ELISA (HAMA, commercially available from Medac, Germany; CA125, commercially available

15   from Centocor, USA). Seventy-five patients were tested for anti-idiotype antibodies (Ab_2) to Alt2 by ELISA (commercially available from AltaRex Corp., USA) and anti-CA125 antibodies by ELISA (AltaRex; see also Schultes *et al.*, Cancer Immunol. Immunother. 46: 201 (1998)). For 17 patients, peripheral blood mononuclear cells (PBMC) were available before and after injection.

20   To determine how quickly the injected Alt2 antibody could form immune complexes with free CA125, CA125 was captured from serum samples (that were obtained at various time points after MAb Alt-2 injection) with the anti-CA125 antibody MAb-B27.1, which recognizes an epitope distinct from MAb Alt-2. After washing, tubes were incubated with ^{125}I-MAb-Alt-2 or ^{125}I-MAb OC125. This procedure allows for

25   detection of CA125 bound MAb Alt-2 in the serum, indicated by decreased tracer binding of ^{125}I-MAb Alt-2 in comparison to preinjection samples as well as decreased binding in comparison to ^{125}I-MAb OC125. As shown in Figures 10A and 10B, MAb-Alt-2 was found to form complexes with CA125 within 30 minutes of injection of the antibody. These complexes cleared very slowly from circulation at the first MAb Alt-2 injection

30   (see right half of Figure 10B). The results show that MAb Alt-2 can bind to CA125 in

circulation and form long circulating immune complexes. Consequently there is an opportunity of such complexes to be taken up by the immune system and undergo processing and presentation to T cells.

In addition, PBMC of the antibody-injected patients were analyzed for T cell

5   proliferation to CA125 in a standard ^{3}H-thymidine uptake assay without *in vitro* sensitization (for assay methods see, *e.g.*, Current Protocols in Immunology, ed. John E. Coligan, John Wiley & Sons, Inc. 1993; Current Protocols in Molecular Biology, eds. Ausubel *et al.*, John Wiley & Sons, Inc. 2000). The results are shown in Figures 11A-13B.

10   Induction of humoral (Pearson r=0.8335, p<0.001) and cellular (p=0.037) anti-CA125 responses showed a correlation with the amount of circulating CA125 antigen present at the time of Alt2 injection (Figures 11A-11C). Neither B nor T cell responses specific for CA125 were detected in the pre-injection samples. Analysis of the patients' anti-CA125 antibodies revealed that they were directed against multiple epitopes of

15   CA125 (Figure 12). This analysis was done by inhibiting human α-CA125 antibody by various α-CA125 monoclonal antibodies that specifically bound to distinct epitopes on CA125.

In addition, survival from the time of first antibody injection correlated with the generation of anti-CA125 antibodies (median survival 22.9 v. 13.5 months, p=0.0089;

20   Figure 13A) and generation of CA125-specific T cells (84 v. 13.2 months, p=0.0202; Figure 13B) after Alt2 injection for anti-CA125 responders v. non-responders. These results demonstrate the following. The levels of circulating CA125 at time of Alt2 injection showed influence on the frequency and amount of immune responses induced to CA125 after injection of the antibody. Patients' anti-CA125 antibodies were found to be

25   multi-epitopic (See Figure 12). As Figures 13A and 13B show, survival after the first injection of Alt2 (plotted in Kaplan-Meier curves) was significantly longer in patients with CA125-specific antibodies (increase in titer > 3 times pre-injection value), and in patients with CA125-specific T cells (SI>1.5) (log-rank test, p<0.01 and p<0.05 respectively).

30

Example XII

Effect of complex formation on antigen presentation

CA125 was purified from tissue culture supernatants of NIH:OVCAR-3 cells (commercially available from AltaRex Corp.) and PSA was purified from human seminal

5   plasma (commercially available from Scripps, La Jolla, CA) using standard procedures. Photoactivated Alt2 antibody was as described previously (see Example VII). The anti-PSA antibody, Alt6 (commercially available from AltaRex Corp.), is a mouse IgG1 that binds to the region of amino acids 135 to 150 of PSA. HAMA was as described previously (see Example VI). Human dendritic cells were prepared from buffy coats by

10 Ficoll-Hypaque and negative selection with anti-CD3, CD16 and CD19, followed by anti-mouse-IgG magnetics beads (Dynal). Cells were cultured in 1000 U/ml GM-CSF and 1000 U/ml IL-4 for 4 days.

Murine macrophages were isolated from the peritoneal cavity of Balb/c mice. Specific B cells were isolated from immunized mice by panning on antibody-coated petri

15 dishes. Dendritic cells were loaded with antigen, antibody, or antigen-antibody complex at day 4 and matured with 10 ng/ml TNF-α and 50 U/ml IFN-α 4 hours later. Two stimulation rounds were performed before analyzing the cells for intracellular IFN-γ staining for either CD4 and CD8 T cells or for the release of IFN-γ into the culture supernatant.

20 The results are shown in Figures 14A-14B. These results show that antigen complexed with specific antibody can be preferentially presented by professional APC such as macrophages (Figure 14A) and B cells (Figure 14B). Results from the antigen-antibody system with PSA and CA125 in dendritic cells support an enhanced presentation of the extracellular antigen on MHC class I when offered as an immune complex (see

25 Figures 6, 8A, and 8B). Also, these results further confirmed the results shown in Table IV (above); namely that while mouse IgG1 alone binds weakly to dendritic cells (Figure 2), the binding is enhanced by binding of specific antigen, and substantially enhanced in the presence of HAMA (Figures 3A-5B). Finally, these results demonstrate that dendritic cells present immune complexes better than non-complexed antigen (Figures 6, 8A, and

30 8B) and, upon repeated stimulation, the immune complexes shifted the immune response

from a helper (CD4+) to a cytolytic (CD8+) T cell response (Figures 6 and 8B), indicating presentation of antigen-derived peptides on both MHC class I and class II.

5   Example XIII

                Ex vivo therapeutic treatment

A human-PBL-SCID/BG mouse model is used generally as described in Schultes _et al._, Hybridoma 18: 47-55 (1999). Human ovarian cancer cells NIH:OVCAR-NU-3 are passaged through nude mice and maintained at 37°C and 5% CO_2 using RPMI 1640

10   medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum Life-Technologies, Gaithersburg, MD) SCID/BG mice are obtained from Taconic (Germantown, NY). Tumors are developed in the SCID/BG mice by subcutaneous injection of 4 x 10^6 NIH:OVCAR-NU-3 tumor cells and three weeks incubation. Dendritic cells are loaded with CA125 antigen or with CA125 antigen and Alt2 antibody

15   as described in Example III, with or without HAMA. The combination is then administered to the mouse either intraperitoneally or intravenously. This treatment is repeated every 2 to 3 weeks. Tumor burden and days of survival are monitored. It is expected that the dendritic cells loaded with the antigen-antibody complex have a greater anti-tumor effect than the dendritic cells loaded with antigen alone, and that the greatest

20   effect is observed when the dendritic cells loaded with the antigen-antibody-HAMA complex are administered.

                    Example XIV

          In vivo Efficacy of an Antigen-Specific

25   Monoclonal Antibody in Murine Animal Model

The efficacy of the Alt1, Alt2 and Alt2 monoclonal antibodies (MAb) _in vivo_ and the role of antibody-antigen complexes in the induction of an immune response and anti-tumor responses were studied in murine animal models. The immunization of mice with Alt1, Alt2 and Alt6 MAb was studied to determine whether Alt1, Alt2 and Alt6 can (a)

30   induce a specific immunity against the specific antigen, MUC1, CA125 or PSA,

respectively. (b) protect the mice against subsequent tumor challenge; and (c) eradicate established tumors and/or increase survival.

These experiments were performed using DBA and Balb/c and human PBL-SCID/bg mice which allowed one to choose the best animal model for future

5   experiments. Five groups of mice were immunized respectively with MAb, control MAb, and MAb/antigen complexes. Antigen and PBS controls were studied. The immunization and tumor induction procedures were identical to the ones used for previous studies. Survival curves were plotted using the Meir Kaplan Algorithm. Comparison of responses between various groups were analyzed using standard statistical procedures.

10   Specifically, to do these studies, the treatment groups described in Table V were set up using SCID/bg mice:

Table V

Group	Mice Number	Treatment
1	9	phosphate buffered saline (PBS) i.v.
2	9	MOPC-21 i.v.
3	9	Alt-6 i.v.
4	9	Alt-6 plus PSA i.v.
5	8	PSA i.v.
6	8	Alt-6 s.c
7	8	MOPC-21 s.c.

i.v. (intravenous) injection: 50 µg of antibody and/or 10 µg of PSA
i.p. (intraperitoneal) injection: 100 µg of antibody and/or 20 µg PSA
15   s.c. (subcutaneous) injection: 50 µg of antibody

Groups 1-5 received the first two injections intraperitoneally., and the next four intravenously
Groups 6-7 received the first two injection intraperitoneally, and the next four
20   subcutaneously with the adjuvant Quil A.

The treatment schedule was as follows in Table VI:

25

Table VI

Day	procedure	comments
1	PBL (1×10^7 human PBL/mouse) i.v. plus immunization, i.p.	PBL (peripheral blood leukocytes) from one HLA-A2+ donor
8	immunization, i.p. Dose 2	
12	Bleeding for hIgG testing	3×10^6/mouse with 75% Matrigel
18	LnCap (tumor) inoculation s.c.	
21	immunization i.v./s.c. Dose 3	
29	immunization i.v./s.c. Dose 4	
35	immunization i.v./s.c. Dose 5	
42	immunization i.v./s.c. Dose 6	
48	Terminate mice/weigh tumor	When biggest tumor reached 10x19mm

On Day 22, human IgG levels were measured (as mg/ml). As can be seen on Figure 15, those mice injected with PBS only (*i.e.*, Group 1) had the highest level of human IgG antibodies.

To measure successful reconstitution of human PBLs in the mice, serum human IgG was measured. These results are shown on Table VII.

Table VII

Group	Number of mice	Number of mice with greater than 0.1 mg/ml hIgG	Percentage of mice with greater than 0.1 mg/ml hIgG
PBS i.v.	9	5	56
MOPC-21 i.v.	9	7	78
Alt-6 i.v.	9	0	0
Alt-6 + PSA i.v.	9	2	22
PSA i.v.	8	6	75
Alt-6 s.c	8	4	50
MOPC-21 s.c.	8	3	38

All off the mice in all groups grew tumor (*i.e.*, from the LnCap inoculation). These tumors were palpable 17 days after inoculation (*i.e.*, Day 35). The tumors were measured twice a week.

As shown in Figure 16, the mouse group injected with Alt-6 plus PSA i.v. demonstrated the best tumor suppression (Alt-6 plus PSA i.v. versus PSA iv; P=0.0477). The Alt-6 i.v. group also showed tumor suppression (as measured by tumor volume)..

These results demonstrated that best anti-tumor effects were achieved in mice treated with the antibody-antigen complexes.

The humoral immune response was monitored by measuring the serum levels of Ab2 and Ab3 in ELISAs.

The cellular immune response was monitored according to routine laboratory procedures by measuring lymphocyte proliferation.

5

Example XV

Histopathological and Toxicological Studies

The tissue antigen specificity of ALt1 and Alt2 was examined by histopathological reactivity with both normal and tumor human tissues. The degree of heterogeneity in reactivity was recorded. This study was conducted by a commercial

10   organization (Impath Inc.) under GLP conditions.

Acute and subacute toxicity of naked Alt1 and Alt2 was conducted in two species (rat and rabbits) by the division of the animal services at the University of Alberta. There was toxicity observed in acute or subacute studies.

5

Example XVI

Phase I Clinical Trial

Because there was data available on the "safety" of murine antibodies administered to humans, it was not expected that side effects were a dose limiting problem. However, standard criteria such as major organ toxicity and patient symptoms

0   were followed utilizing GCP. As antibody doses did not have to be pushed to toxicity (the maximally tolerated dose) the major outcome became defining the most effective dose of antibody that elicited the desired defined immune response.

The patient population to be studied needed to be immune competent and have the target disease. A group of patients with MUC1 expressing tumor were enrolled in a

5   three-dose Phase I trial with Alt-1

Based on studies with other immune therapies it was expected that the effective dose was in the 1-4 mg dose. Doses of 1, 2, and 4 mg were studied in the stated patient population. Toxicity criteria were monitored along with the immune response (primary endpoint). Evidence of therapeutic activity was detected by monitoring patient MUC1

levels (secondary endpoint). Six patients per dose level were treated. The 2 mg dose was most effective in inducing HAMA, Ab2, anti-MUC1 antibodies and MUC1-specific T cells, and showed the highest incidence of MUC1 serum level stabilization or decrease.

5

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

We claim:

1. A method for treating a patient suffering from a disease associated with an antigen, comprising administering to the patient a composition comprising an antigen associated with the disease, a dendritic cell binding agent specific for the antigen, and a dendritic cell autologous to the patient, wherein the patient administered the composition receives a therapeutic benefit.

2. The method of claim 1, wherein the antigen is complexed to the binding agent.

3. The method of claim 1, wherein the dendritic cell added to the composition is an immature dendritic cell.

4. The method of claim 3, wherein the composition is incubated *ex vivo* under conditions that allow for maturation of the immature dendritic cell prior to administering the composition to the patient.

5. The method of claim 1, wherein the dendritic cell added to the composition is a dendritic cell precursor.

6. The method of claim 5, wherein the composition is incubated *ex vivo* under conditions that allow for maturation of the dendritic cell precursor prior to administering the composition to the patient.

7. The method of claim 1, wherein the patient is a human.

8. The method of claim 1, wherein a CD8+ IFN-γ producing T cell is activated to induce a CTL immune response in the patient administered the composition.

9. The method of claim 1, wherein a CD4+ IFN-γ producing T cell is activated to induce a helper T cell immune response in the patient administered the composition.

10. The method of claim 1, wherein a humoral immune response is activated in the patient administered the composition.

11. The method of claim 1, wherein the dendritic cell binding agent specifically binds to the antigen and binds to an Fcγ receptor on a dendritic cell in the patient administered with the composition, wherein the Fcγ receptor is selected from the

group consisting of an Fcγ Type I (CD64) receptor, an Fcγ Type II (CD32) receptor, and an Fcγ Type II CD16 (FcγRIII) receptor

12.   The method of claim 1, wherein the dendritic cell binding agent is an antibody.

13.   The method of claim 12, wherein the dendritic cell binding agent is a xenotypic antibody to the patient.

14.   The method of claim 13, wherein the xenotypic antibody elicits a host anti-xenotypic antibody response in the patient.

15.   The method of claim 13, wherein host anti-xenotypic antibodies (HAXA) are present in the patient's blood prior to administering the composition.

16.   The method of claim 13, wherein the xenotypic antibody is a murine monoclonal antibody.

17.   The method of claim 16, wherein the murine monoclonal antibody is selected from the group consisting of Alt-1, Alt-2, Alt3, Alt-4, Alt-5; and Alt-6.

18.   The method of claim 13, wherein the composition further comprises human anti-xenotypic antibodies (HAXA).

19.   A therapeutic composition comprising a purified dendritic cell binding agent that is specific for an antigen associated with a disease, a dendritic cell, and the antigen associated with the disease

20.   The composition of claim 19, wherein binding of the dendritic cell binding agent to a receptor on the dendritic cell blocks binding of a natural ligand to the receptor.

THERAPEUTIC METHOD AND COMPOSITION UTILIZING ANTIGEN-ANTIBODY COMPLEXATION AND PRESENTATION BY DENDRITIC CELLS

ABSTRACT OF THE DISCLOSURE

5

Disclosed are methods and compositions for use in immunotherapy. These methods and compositions are particularly useful for exploiting dendritic cells to present an antigen to a patient, particularly where the patient has a disease associated with the antigen. The invention provides methods for treating a patient having a disease

10 associated with an antigen. The methods according to the invention comprise combining *ex vivo* an antigen and an antigen-presenting cell binding agent specific for the antigen, and administering the composition to a patient suffering from a disease associated with the antigen, wherein the patient receives a therapeutic benefit.

15
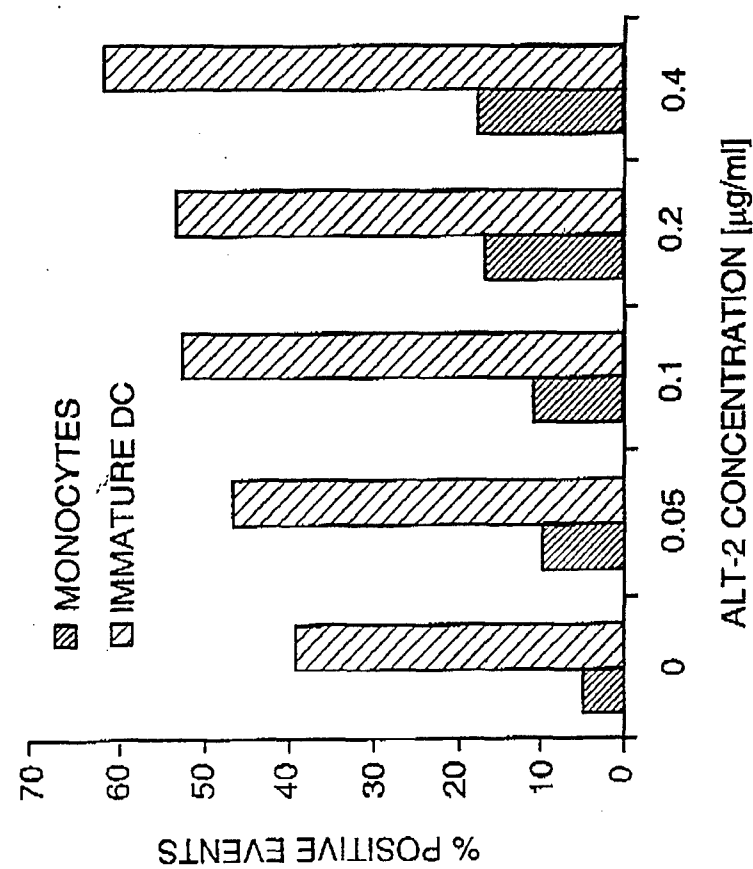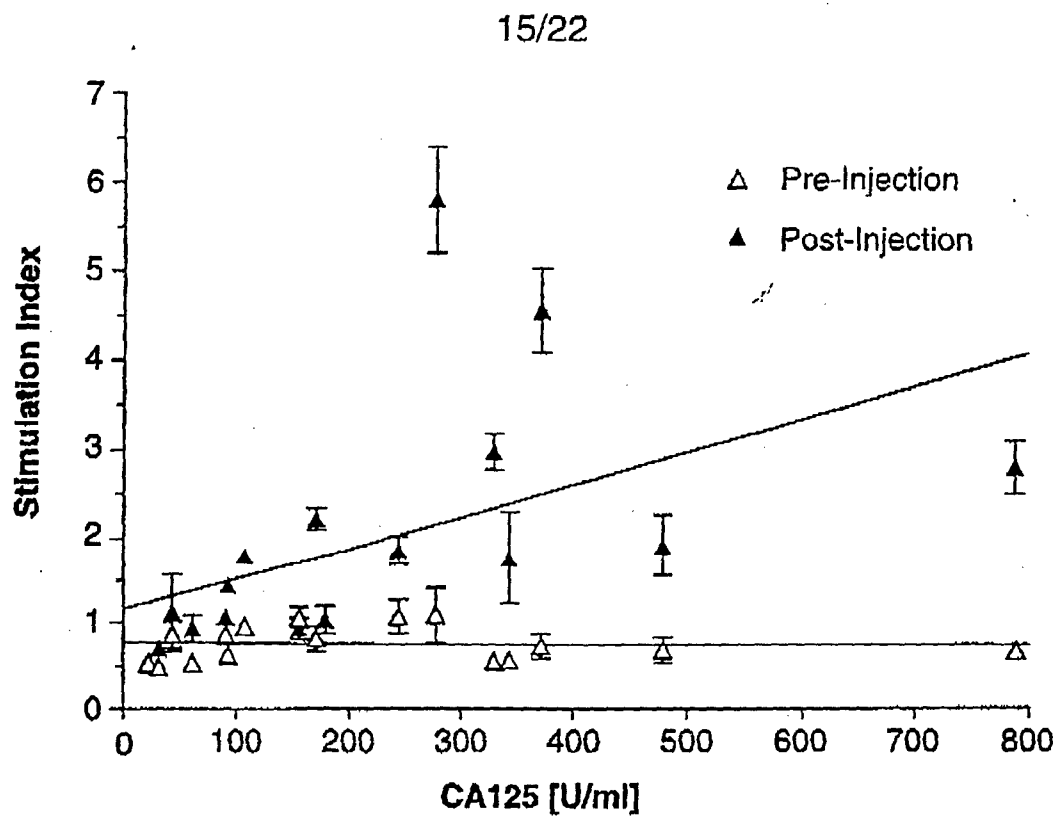
FIG. 1A
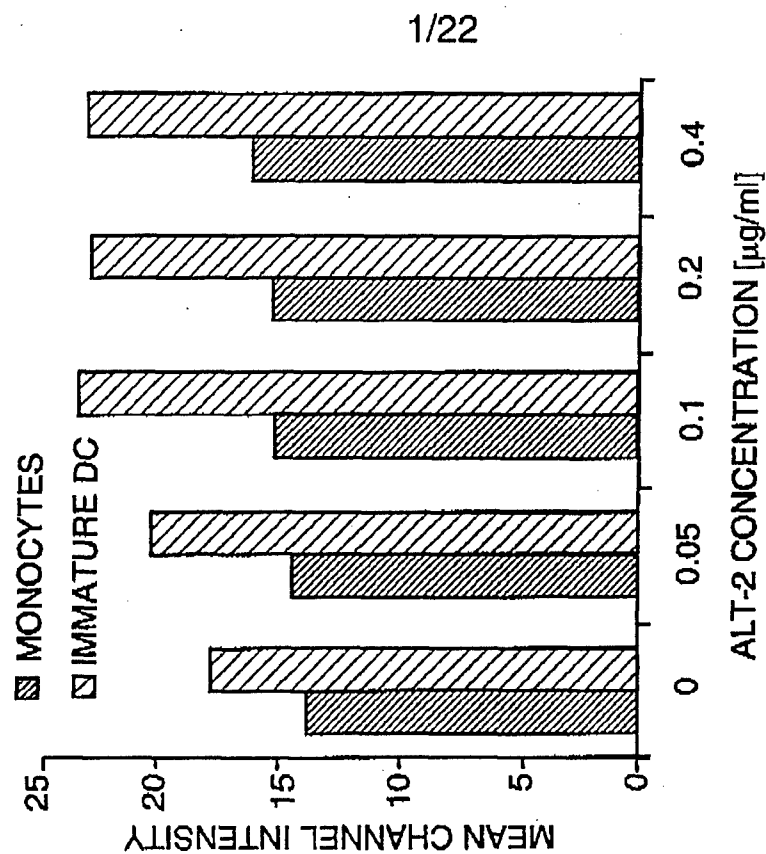
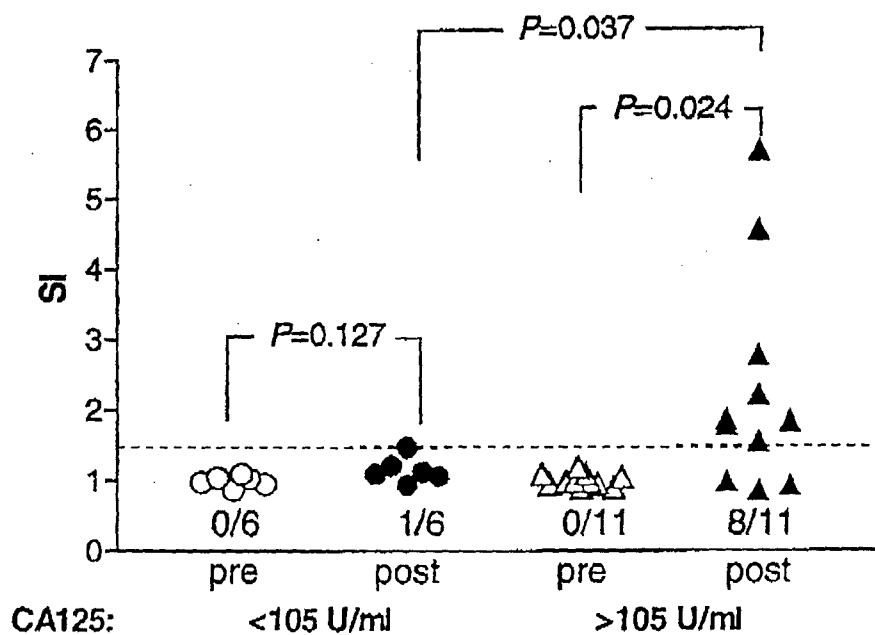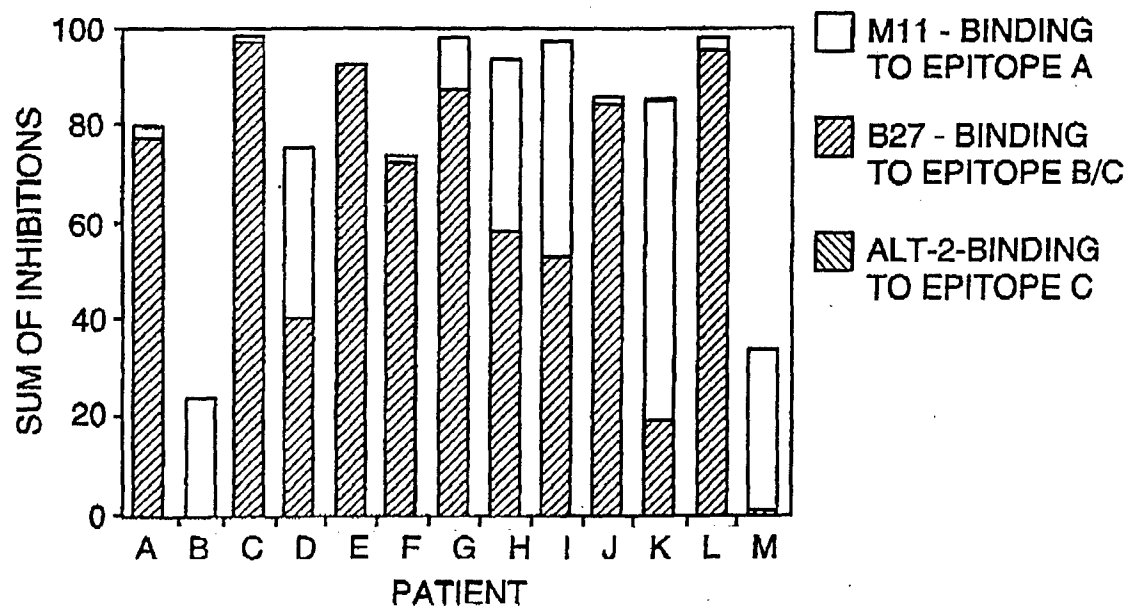FIG. 1B
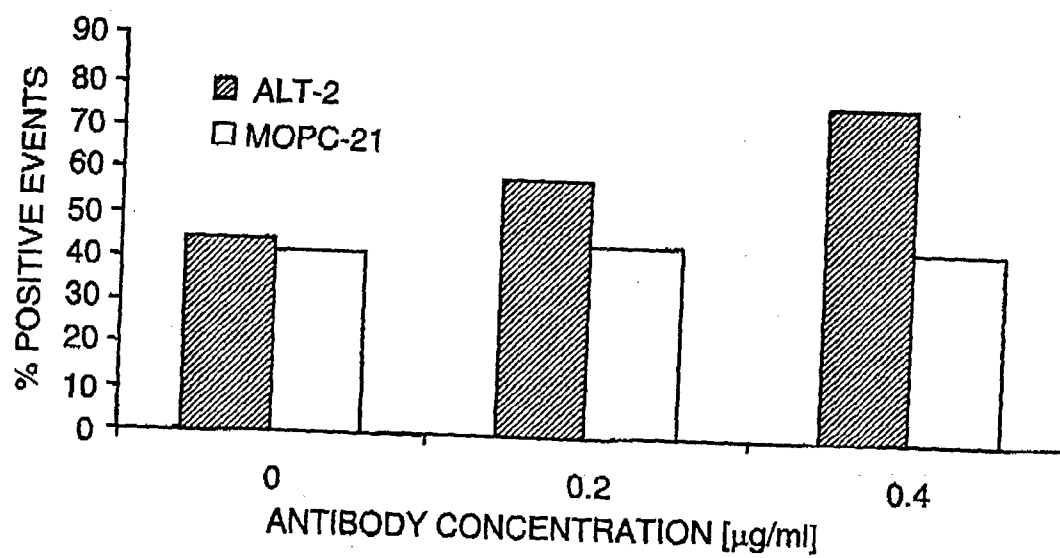
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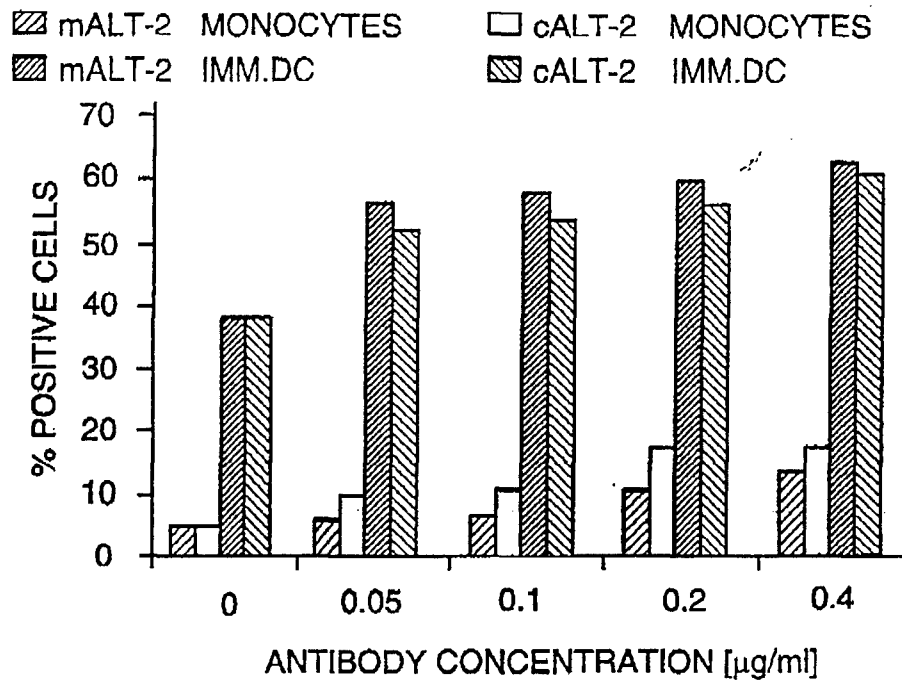
FIG. 3A
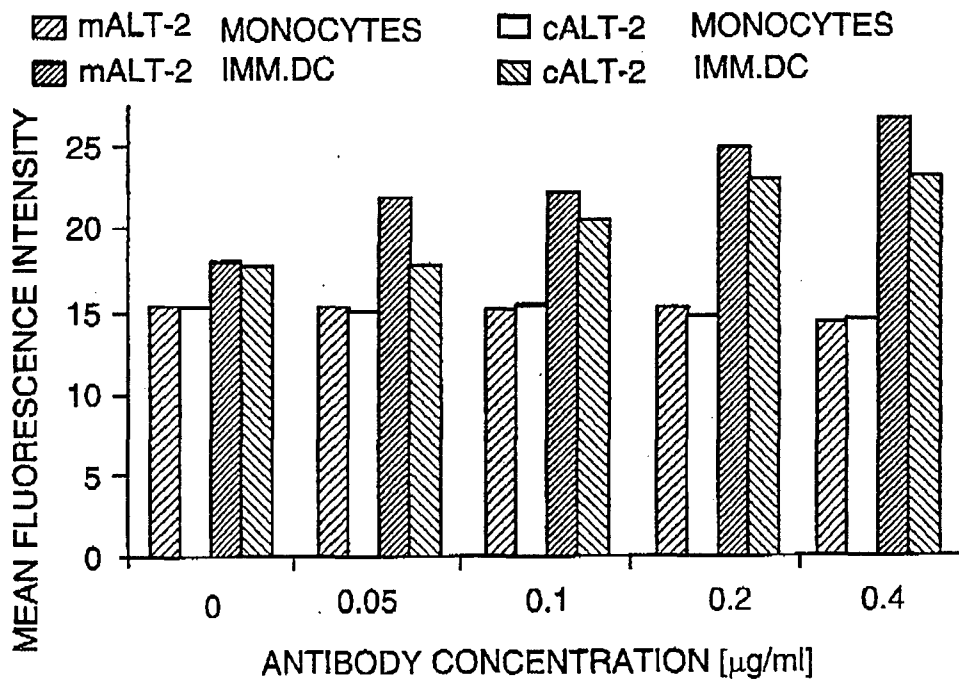
FIG. 3B

COMPLEXATION WITH SPECIFIC ANTIGEN OR HAMA
INCREASES ANTIBODY-BINDING TO DC
(FACScan)
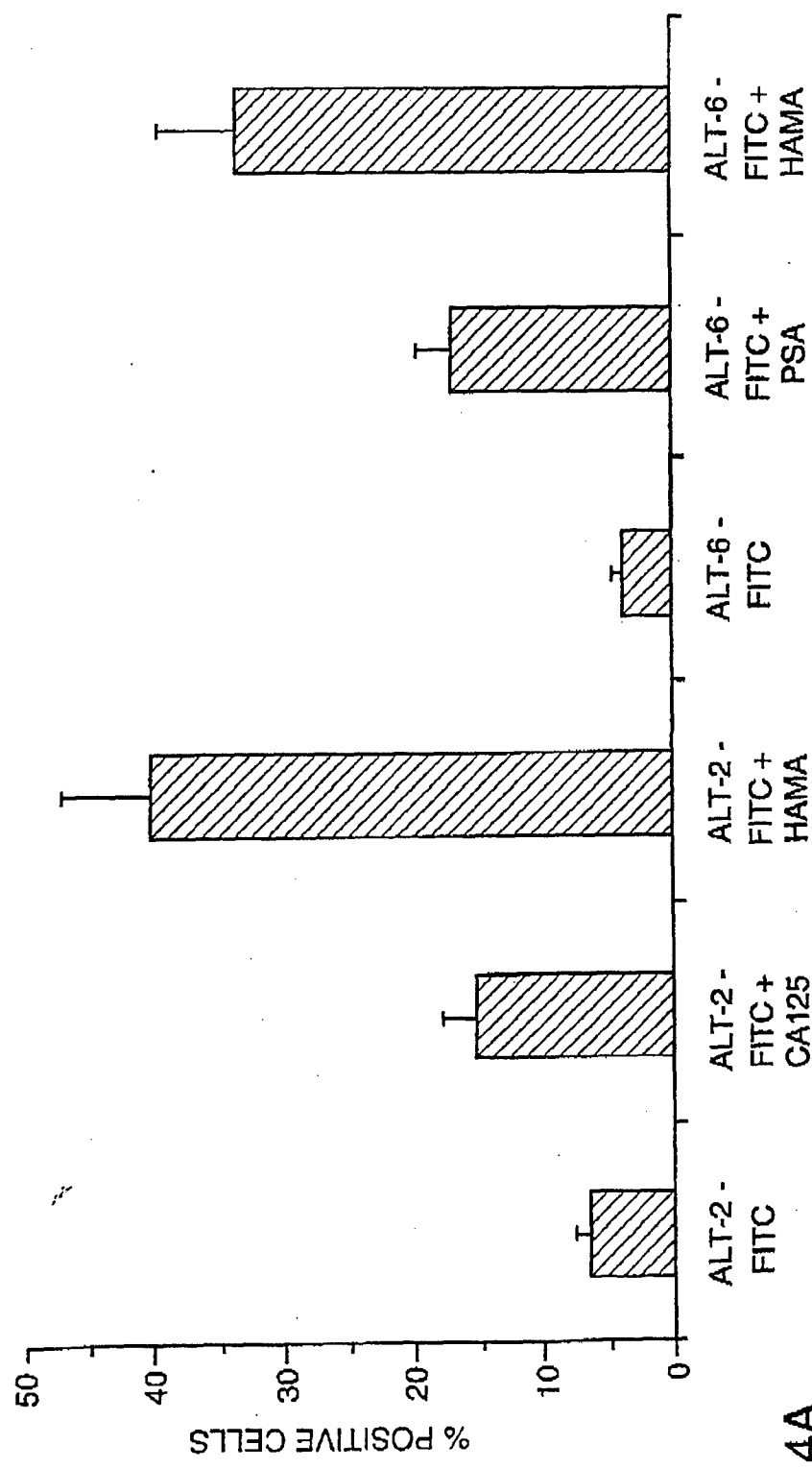
FIG. 4A

CA125-FITC BINDING TO DENDRITIC CELLS IN THE
ABSENCE AND PRESENCE OF MAb-ALT-2

CA 125: 8000 U/ml

ALT-2 CONCENTRATION [µg/ml]
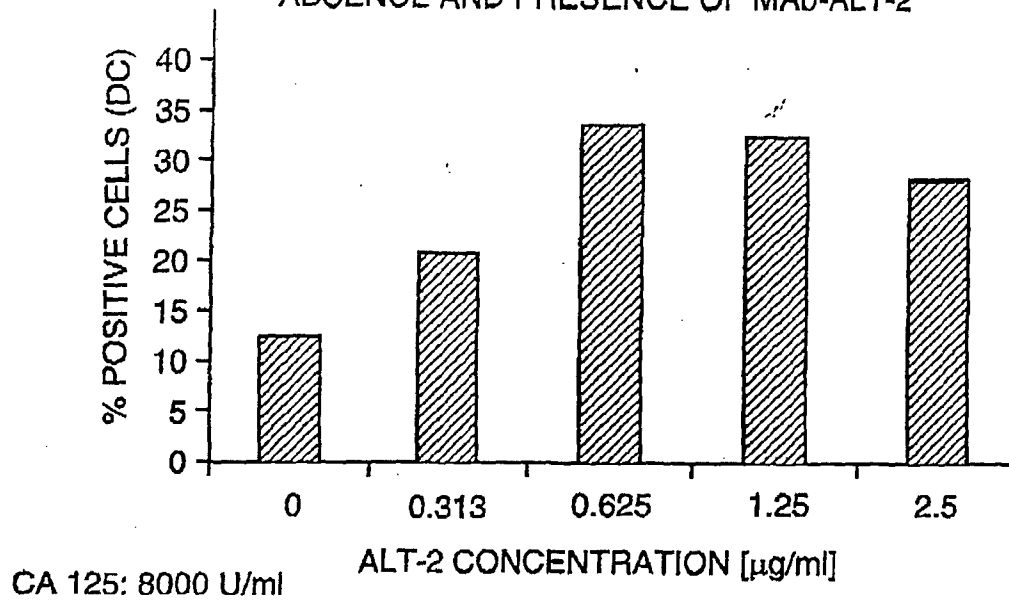
FIG. 4B

BINDING OF ALT-2 -CA125 TO DENDRITIC CELLS IN
THE ABSENCE AND PRESENCE OF HAMA

HAMA CONCENTRATION [µg/ml]

ALT-2: 1 µg/mL, CA 125: 8000 U/ml

FIG. 4C

FIG. 5A

FIG. 5B

FIG. 6

IN VITRO T-CELL ACTIVATION BY MONOCYTE-DERIVED DENDRITIC CELLS
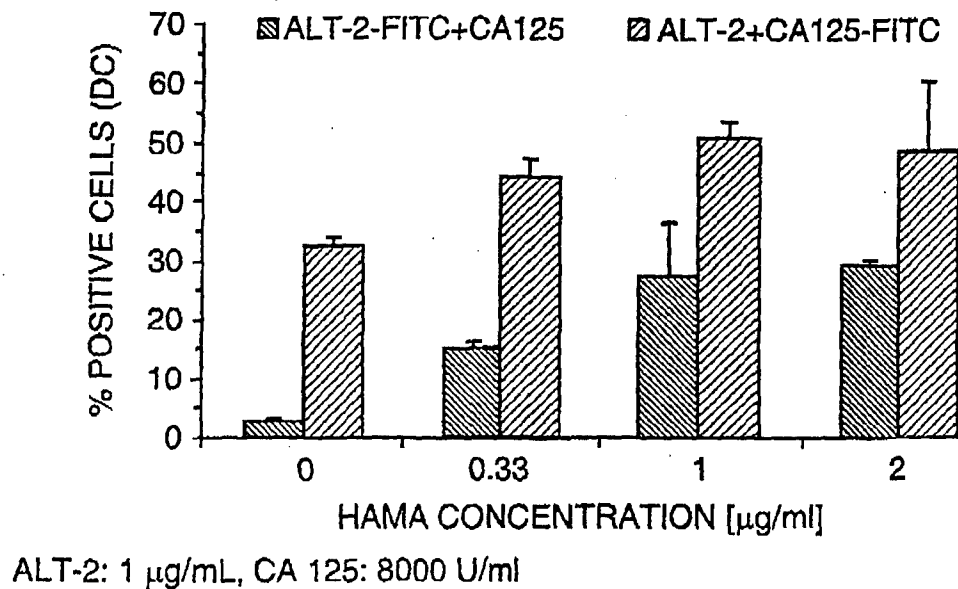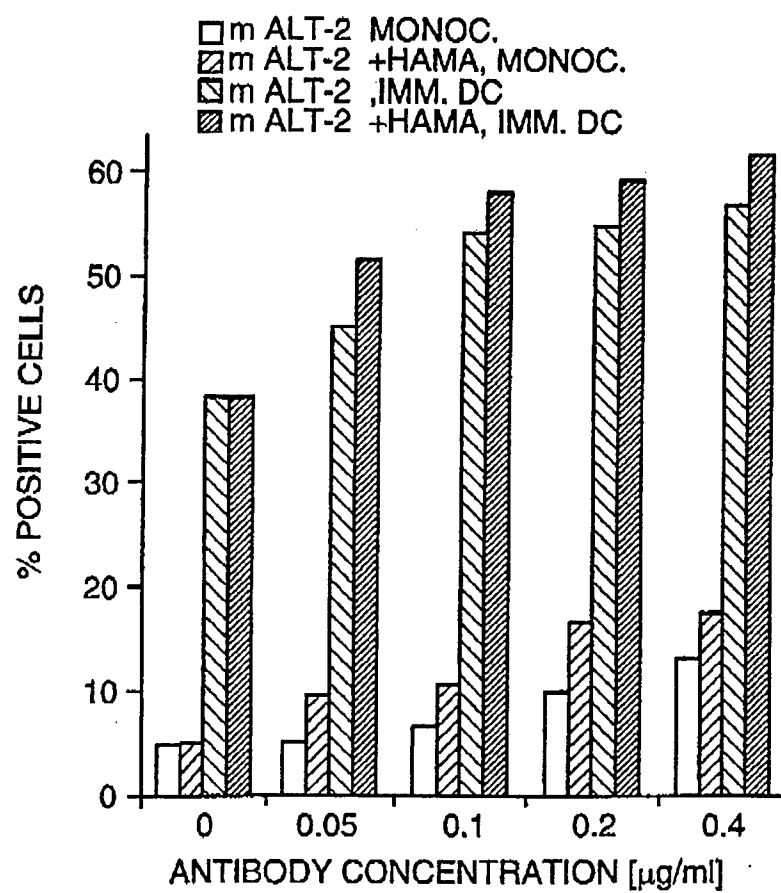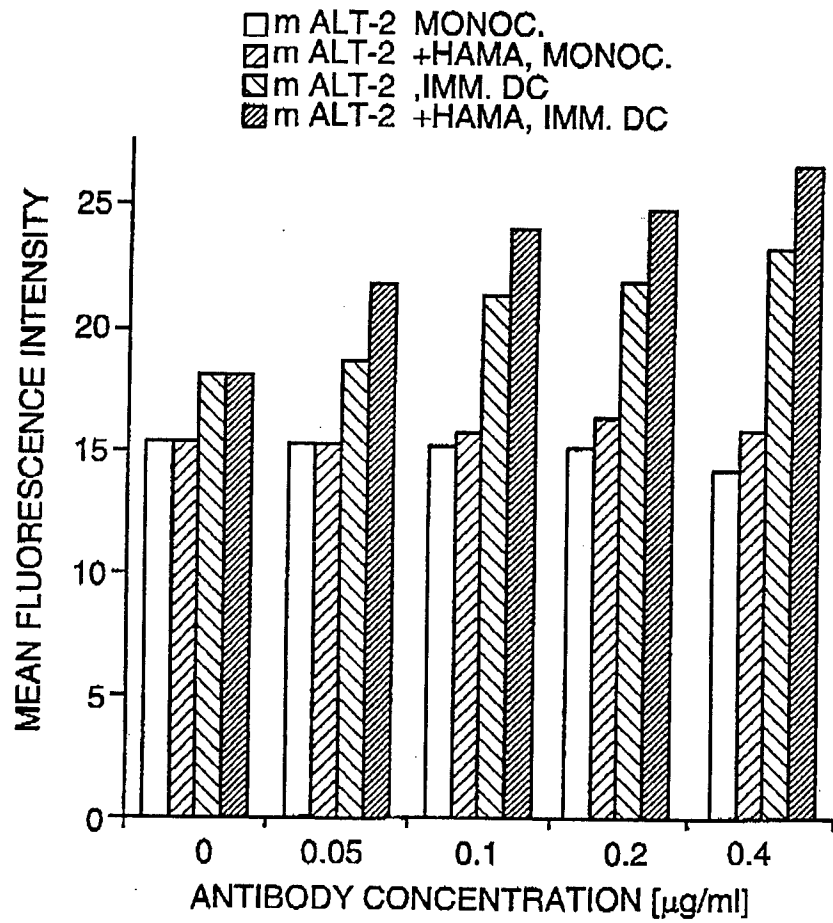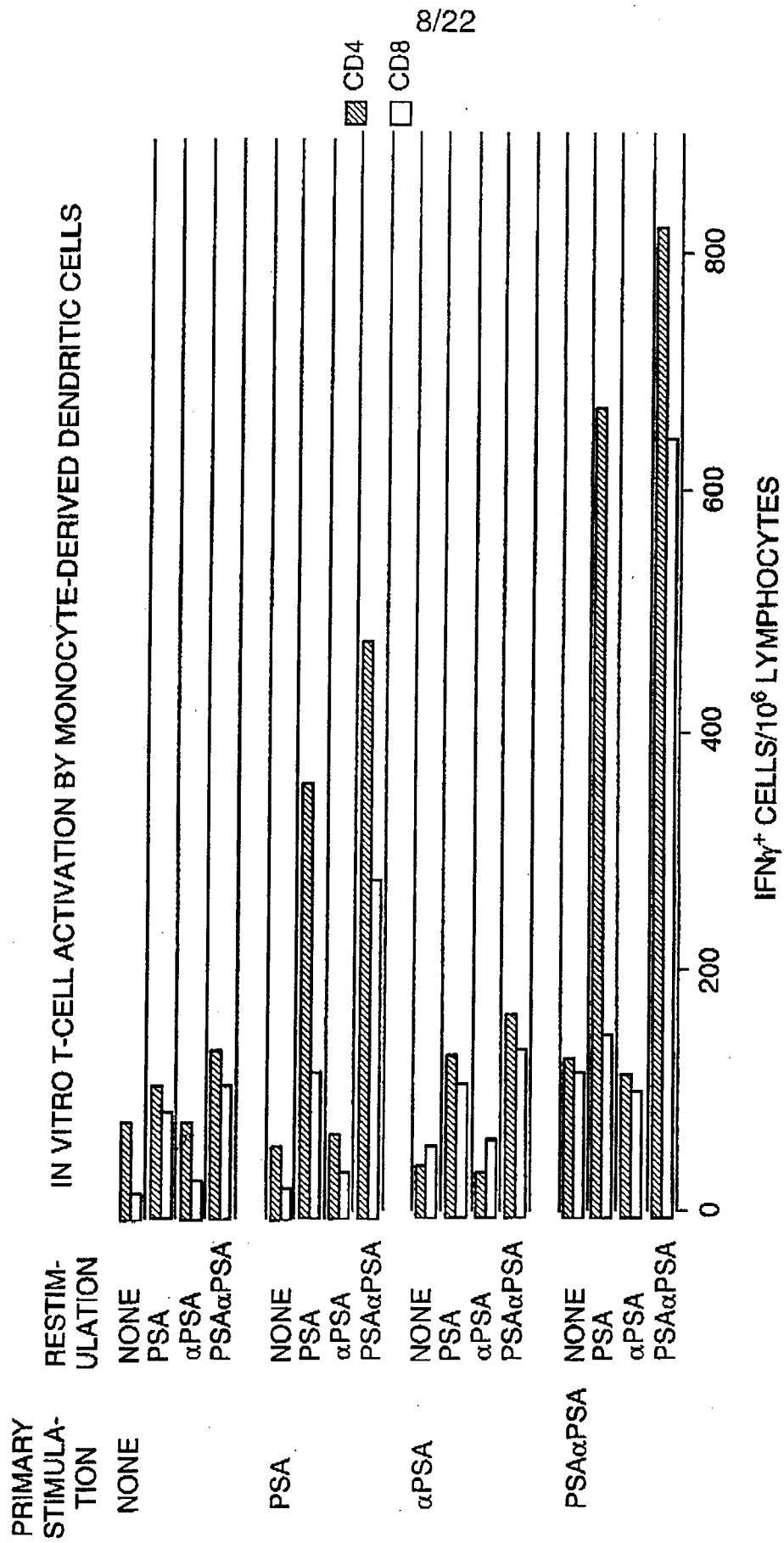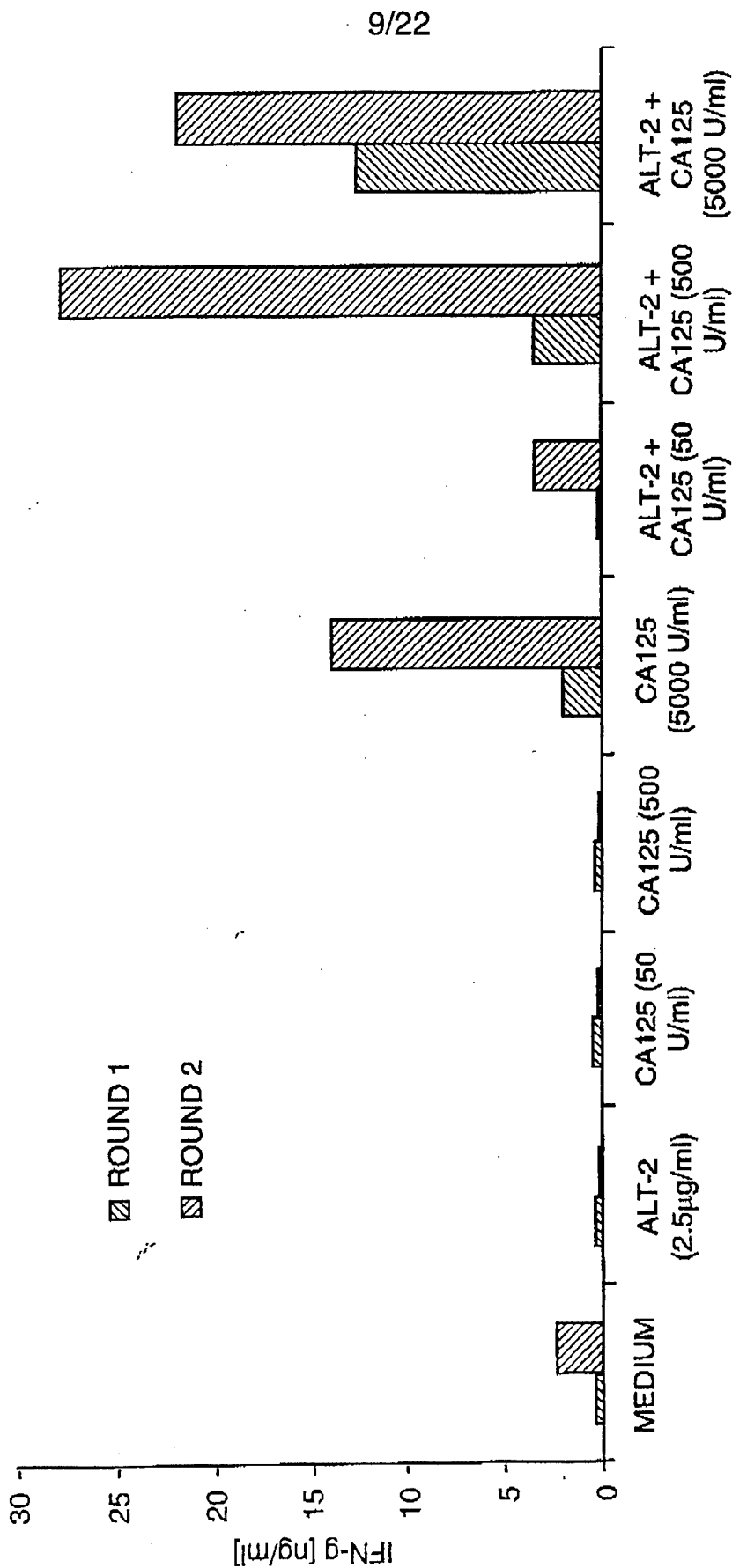
FIG. 7
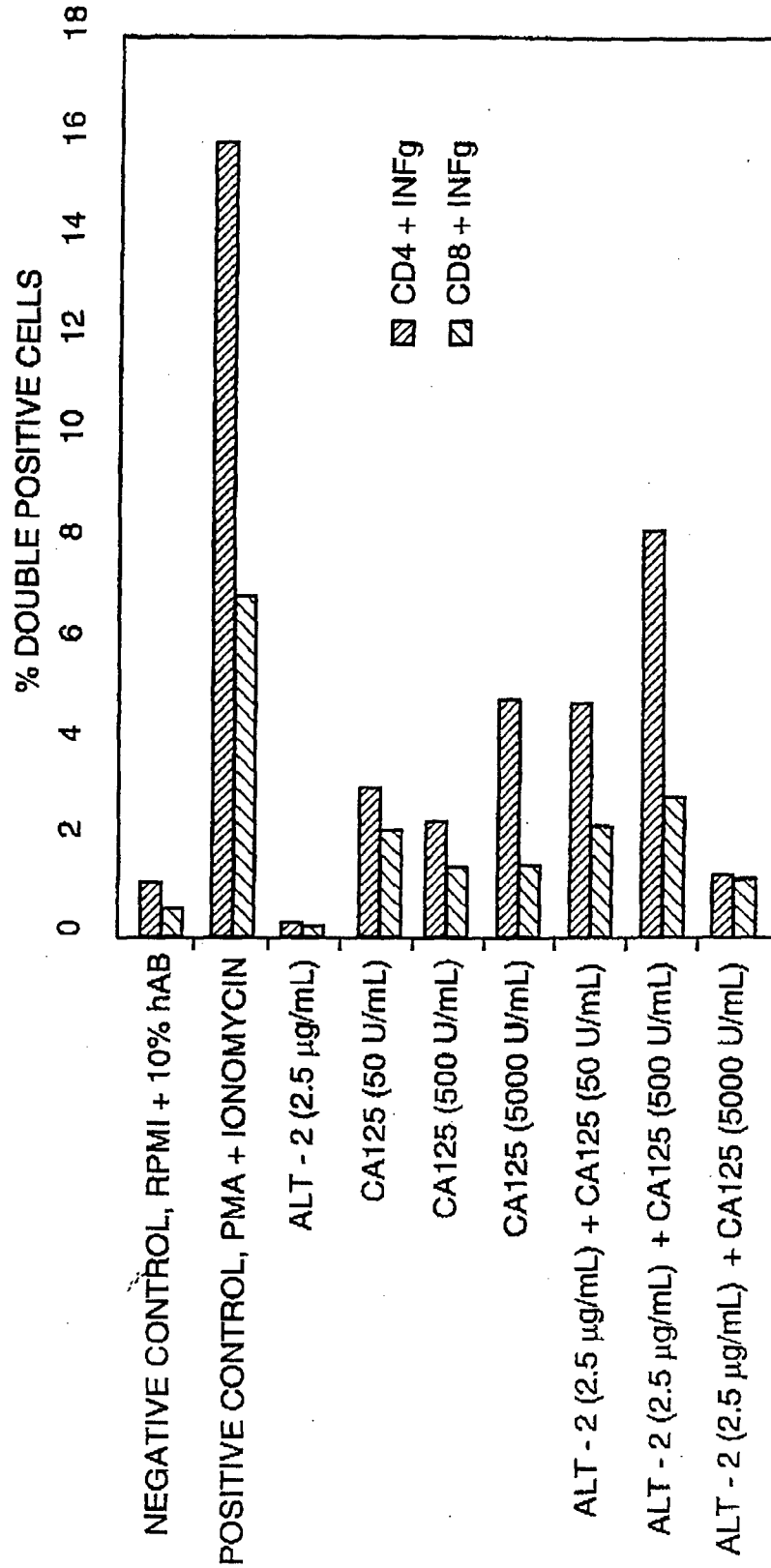
FIG. 8A
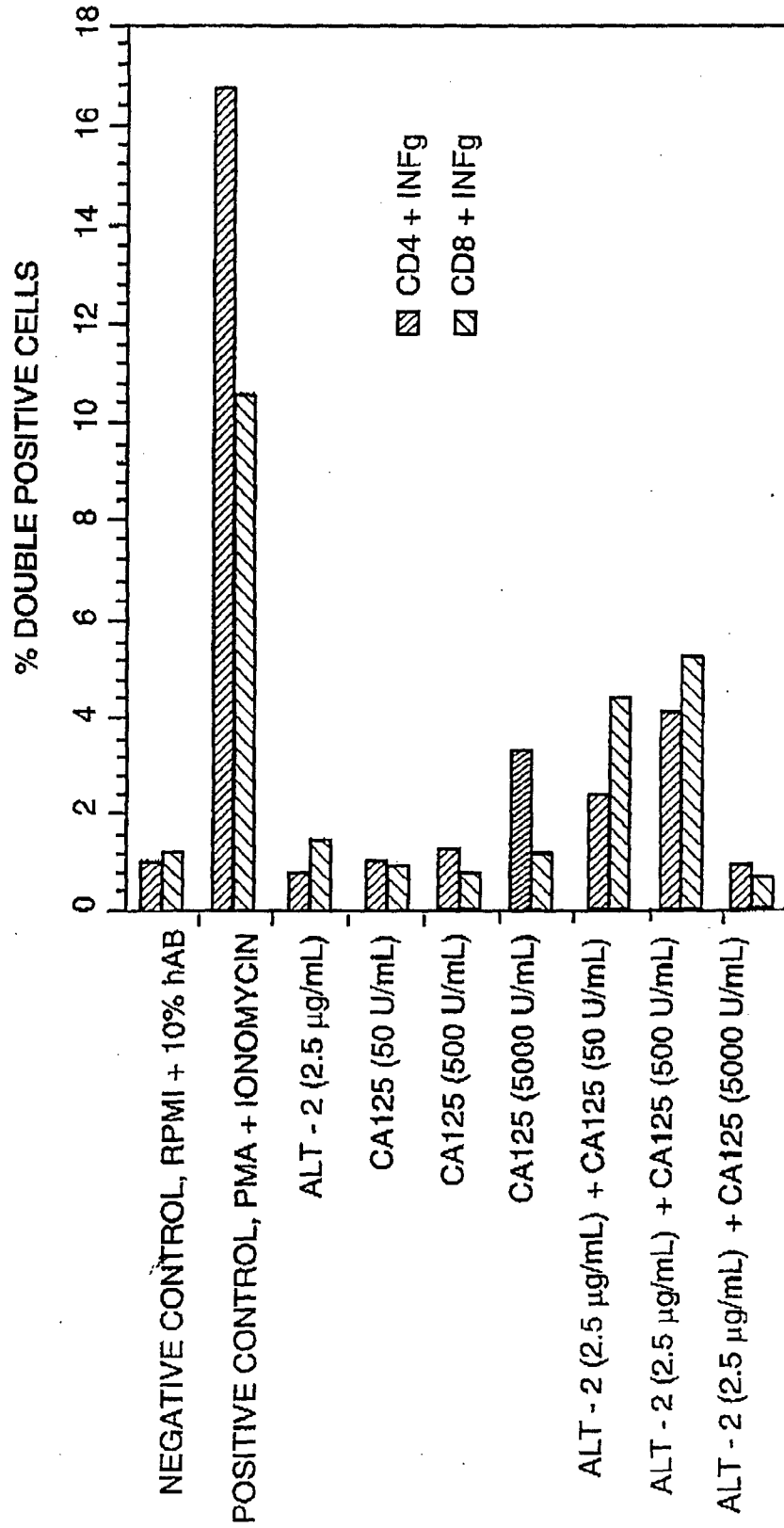
FIG. 8B
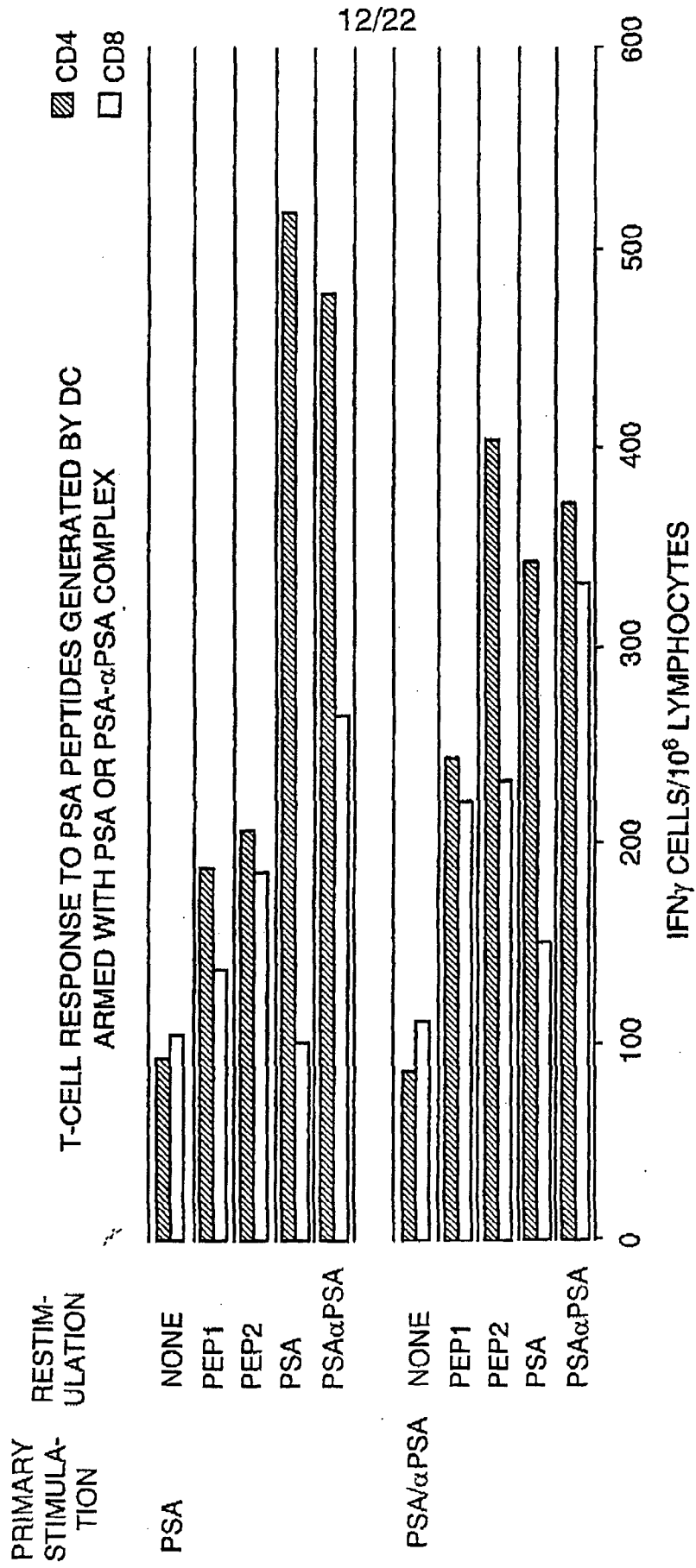
FIG. 9

MAb- ALT-2 FORMS COMPLEXES
WITH CA125 WITHIN 30 MIN. OF
INJECTION OF THE ANTIBODY
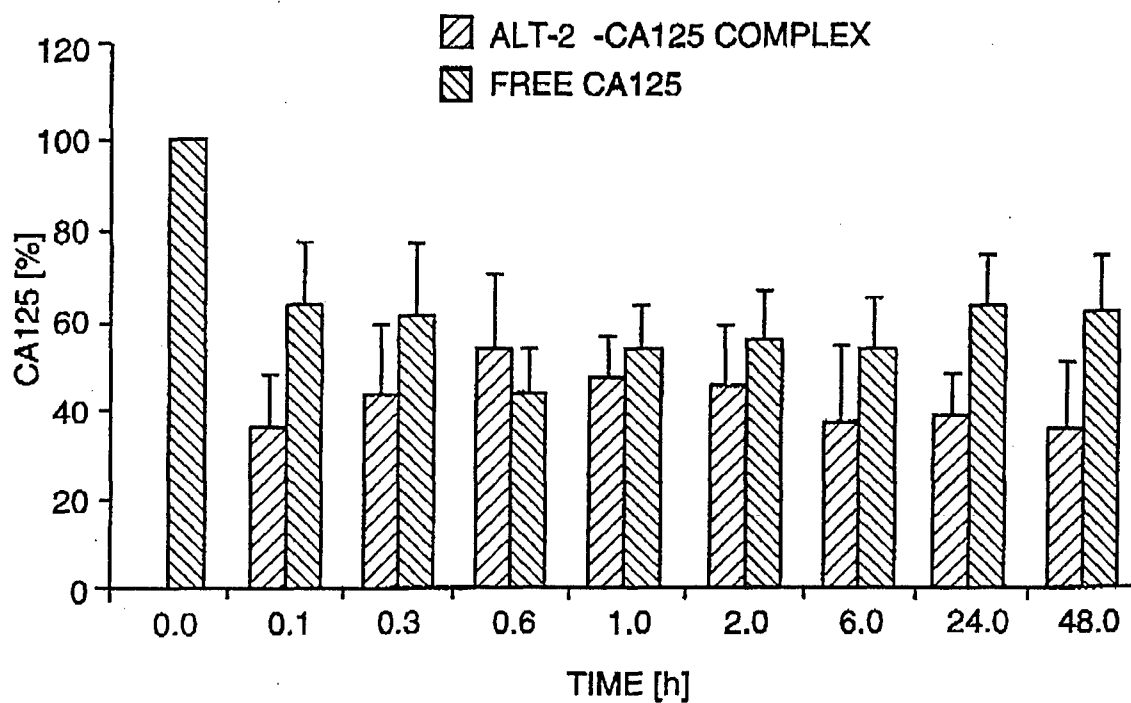
FIG. 10A
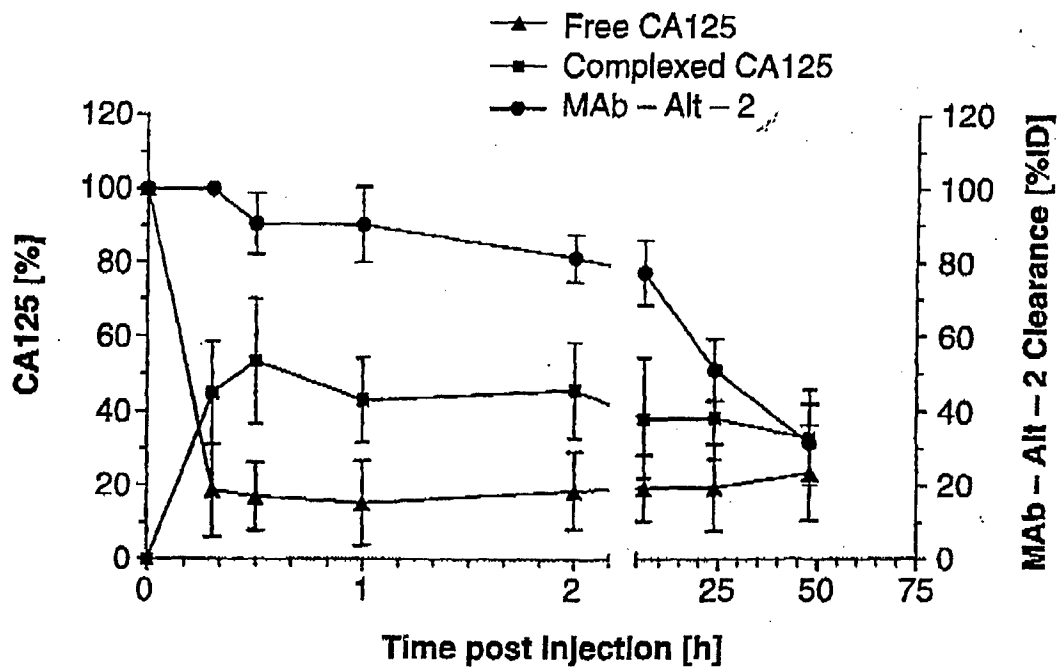
FIG. 10B
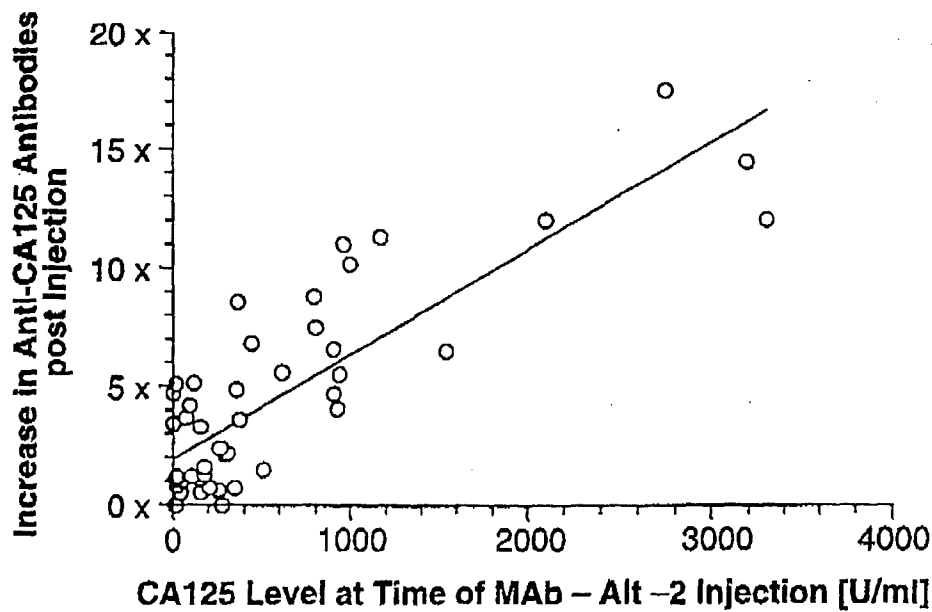
FIG. 11A

FIG. 11B

FIG. 11C

FIG. 12
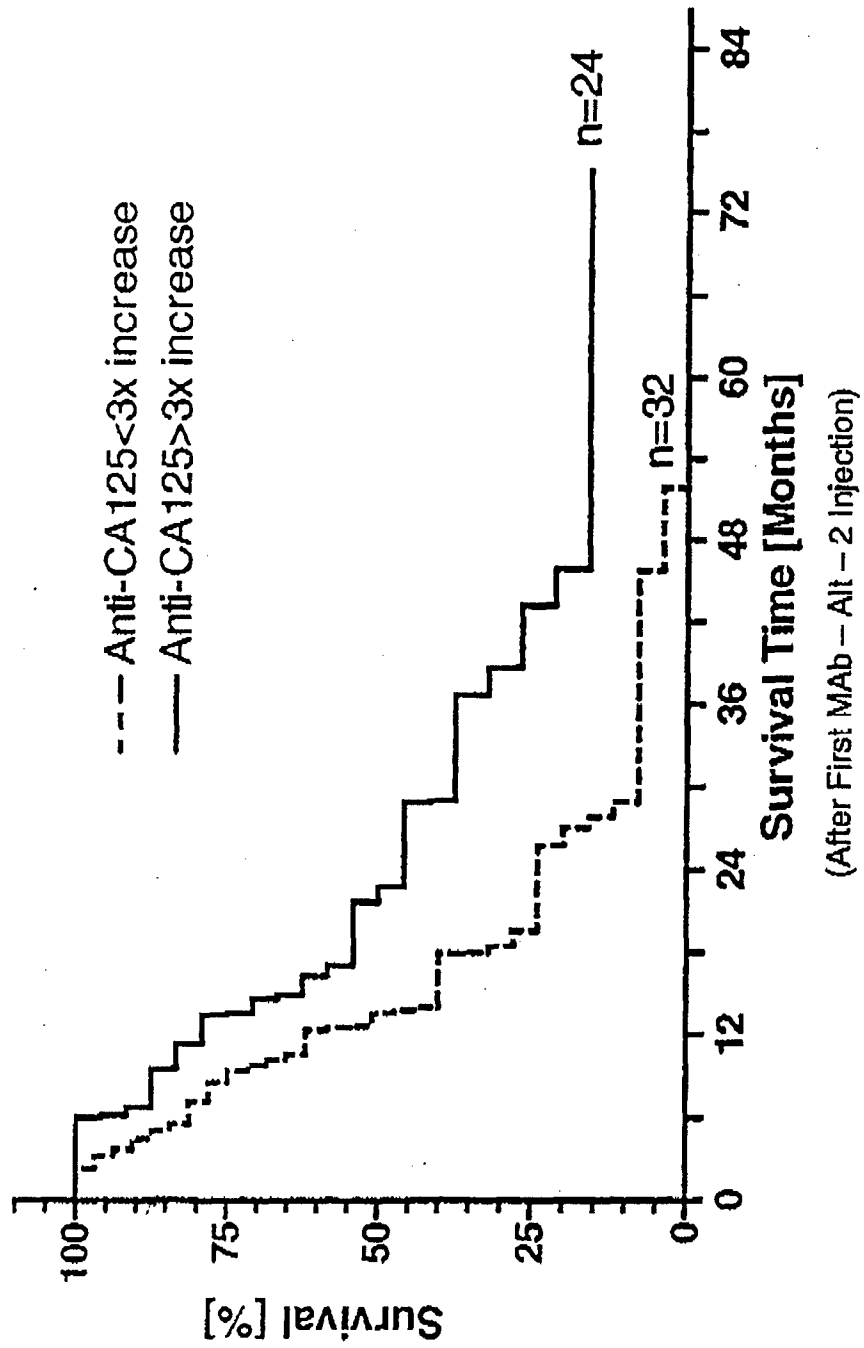
FIG. 13A
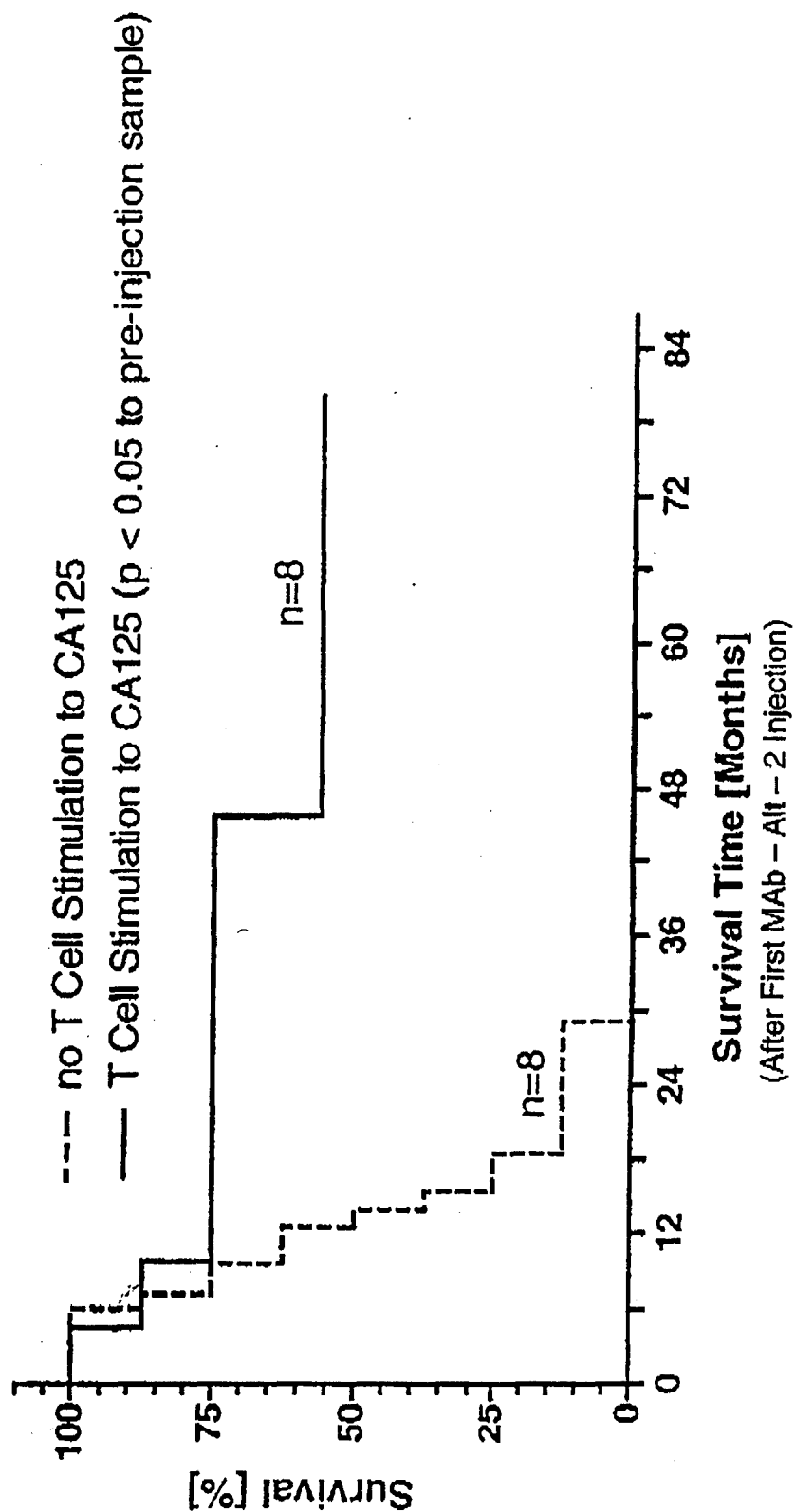
FIG. 13B
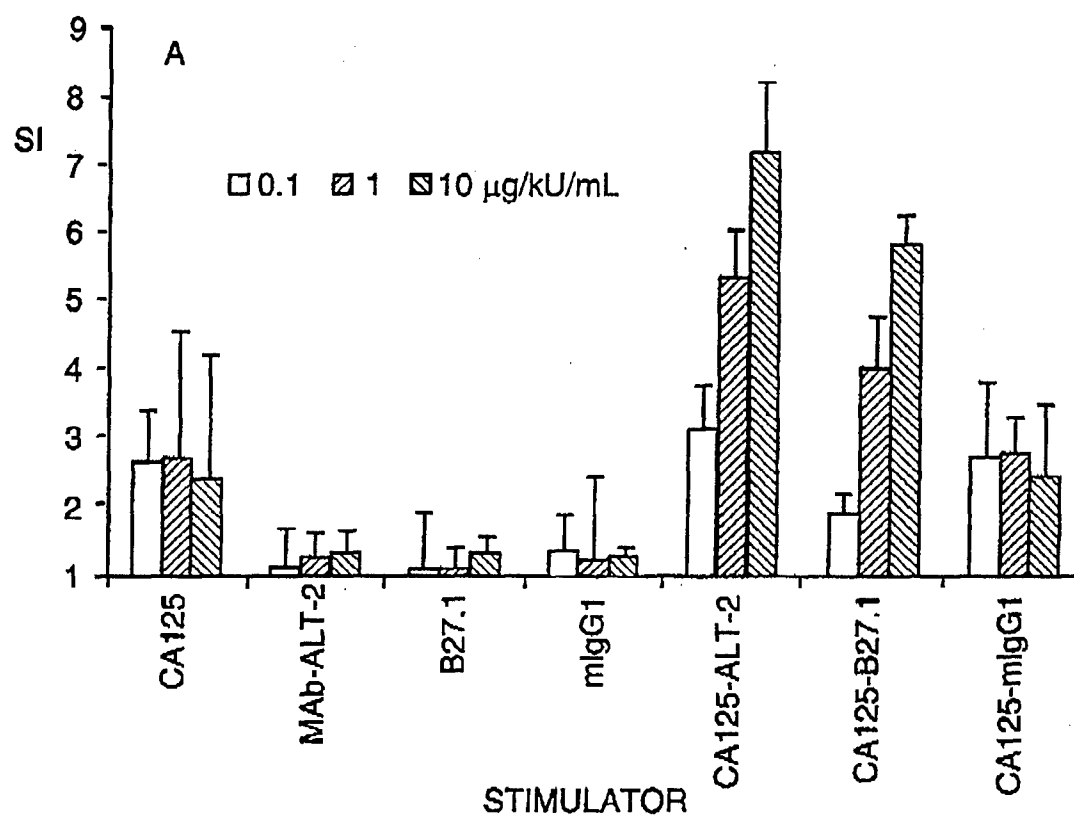
FIG. 14A
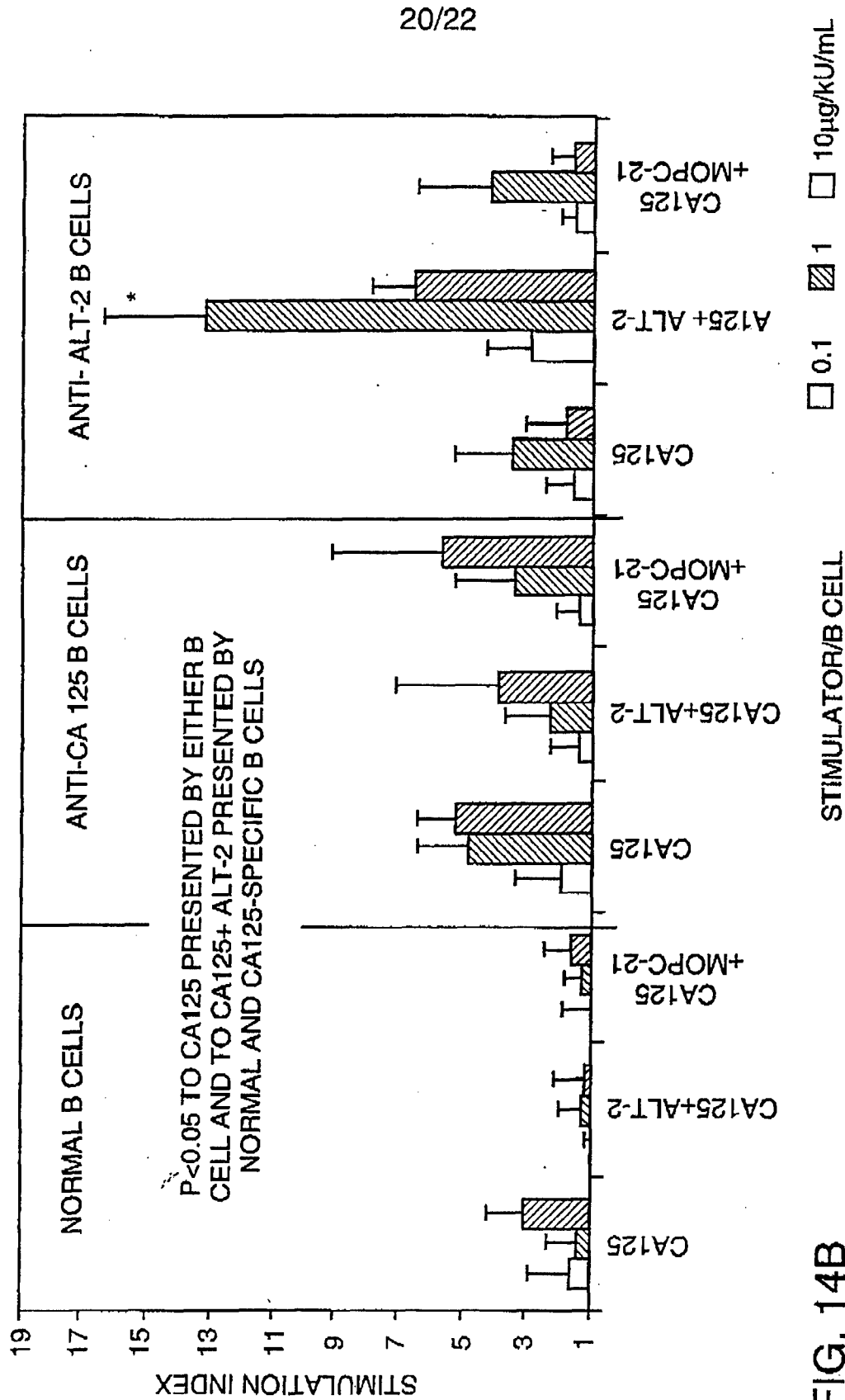
FIG. 14B
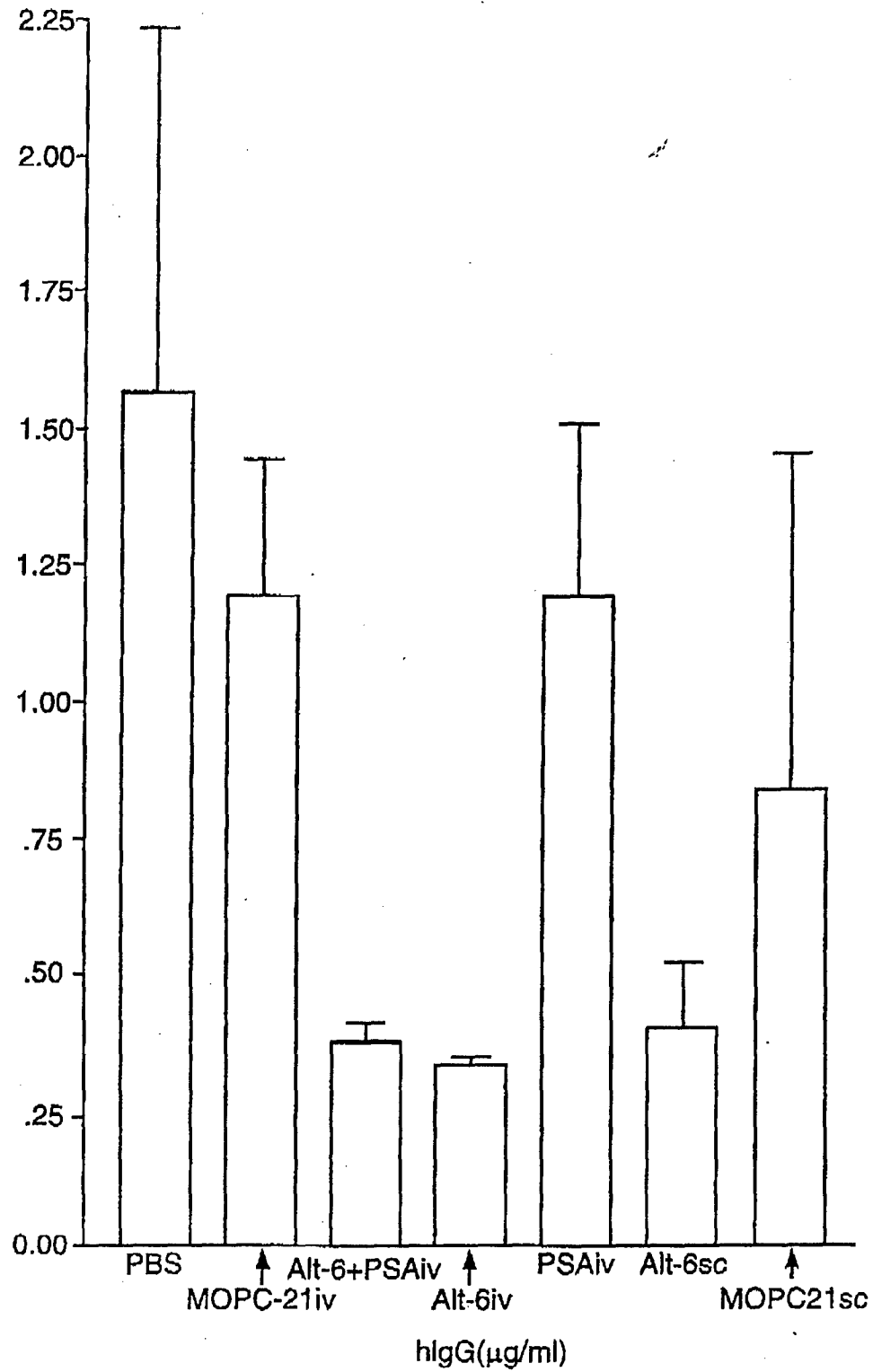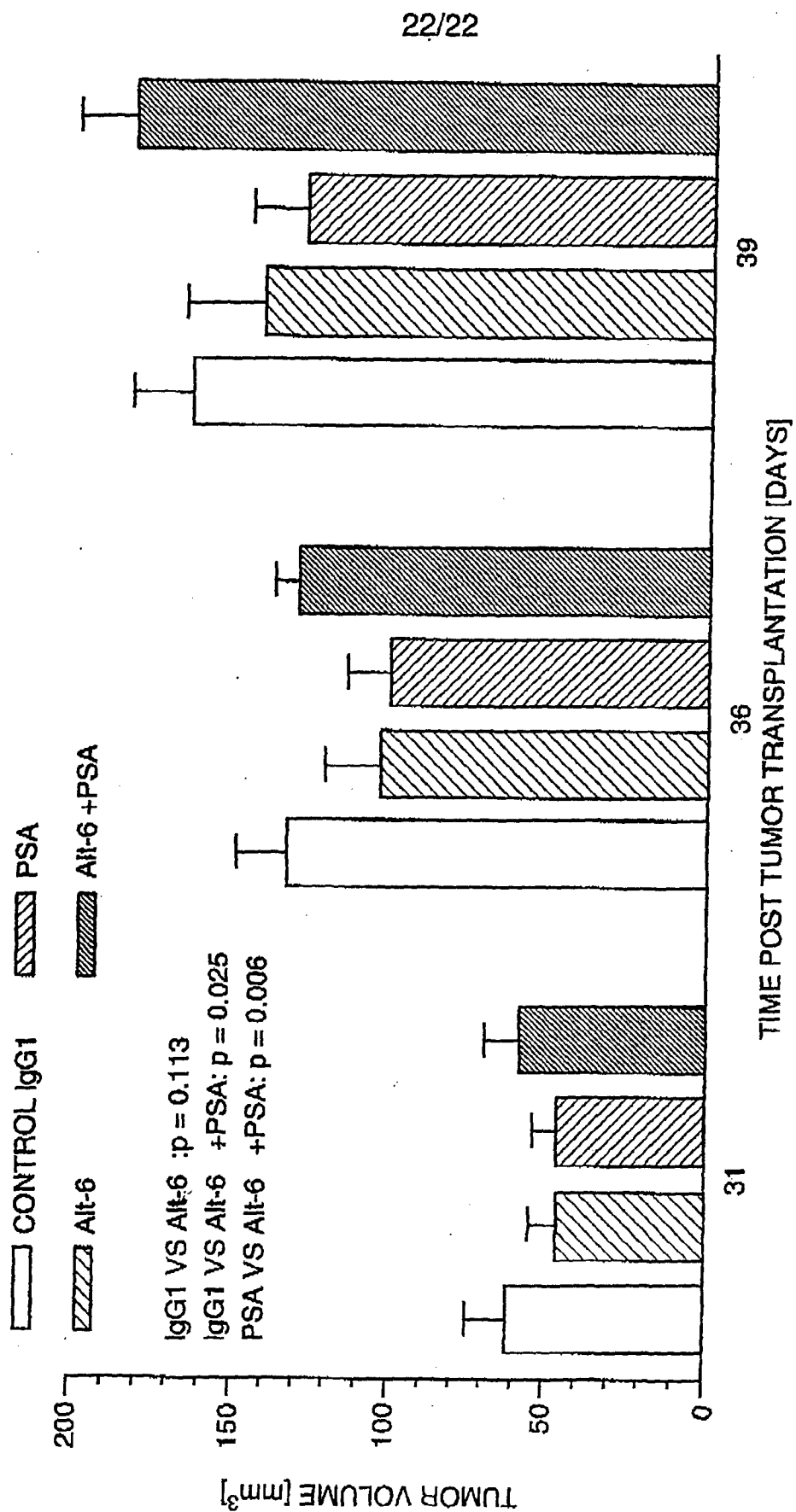
FIG. 15

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FIG. 16

TUMOR VOLUME [mm³]

TIME POST TUMOR TRANSPLANTATION [DAYS]

Alt-6 +PSA VS. PSA: p<0.05

CONTROL IgG1
Alt-6
PSA
Alt-6 +PSA

IgG1 VS Alt-6 : p = 0.113
IgG1 VS Alt-6 +PSA: p = 0.025
PSA VS Alt-6 +PSA: p = 0.006

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)   4 Dec 02

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Encl   PHYLIS M. RINEHART
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